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Characterization of SIP470, a Family 1 Lipid Transfer Protein and its Role in Plant Stress
Signaling

A thesis

Presented to

The faculty of the Department of Biological Sciences

East Tennessee State University

In partial fulfillment

Of the requirement for the degree

Master of Science in Biology

by

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August 2016

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Keywords: ltp12, ltp2, SA, SABP2, SIP470, Lipid Transfer Proteins (LTP), TNS

ABSTRACT

Characterization of SIP470, a Family 1 Lipid Transfer Protein and its Role in Plant Stress Signaling

by

Timothy Ndagi Audam

SIP470, a putative tobacco lipid transfer protein, was identified in a yeast two-hybrid screen to interact with SABP2. SABP2 is a critical role in SA-mediated signaling in tobacco and other plants. *In vitro* studies using purified recombinant SIP470 confirmed that it is a lipid binding protein. In an attempt to determine its role in mediating stress responses, Arabidopsis T-DNA insertion knockout lines lacking SIP470 homolog were used for the analysis. These mutant plants were defective in basal resistance against microbial pathogens. Expression of defense gene *PR-1* was also delayed in these mutant plants. Interestingly, these mutant plants were not defective in inducing systemic acquired resistance. Besides biotic stress, these mutant plants also showed increased susceptibility to abiotic stresses. To directly study the role of SIP470 in tobacco plants, transgenic tobacco lines, with reduced levels of SIP470 expression, were generated using RNAi and transgenic lines overexpressing SIP470 were also generated.

DEDICATION

This work is dedicated to God almighty, for without Him I am nothing.

To my wonderful parents, Mr. and Mrs. Audam for their love and their all-round support.

ACKNOWLEDGEMENTS

I would like to express my profound gratitude to members of my committee, Dr. Dhirendra Kumar, Dr. Ranjan Chakraborty, and Dr. Karl Joplin, for their guidance, support, and constructive criticisms. I would like to especially thank Dr. Dhirendra Kumar for his constant support, tutelage, guidance and for always pushing me to bring out the best in me. I would also like to thank the staff and faculty of the Department of Biological Sciences, ETSU for their kindness and support. I would like to acknowledge the support of my colleagues in the lab, both graduates, and undergraduates including past members. Special thanks to Bal Krishna Chand Thakuri, Behzad Alinejad, Keerthi Ganapenani, and Mackenzie Davenport for helping me out with my experiments whenever I couldn't make it to the lab. I will also like to thank the ETSU School of Graduate Studies for the graduate assistantship. This research was supported by a grant from the National Science Foundation (MCB#1022077) and major RDC grant (RDC 14-021) to DK and funds from the Department of Biological Sciences, ETSU, and Sigma Xi Grant-in-aid of Research Program and William Harvey and Nina M. Fraley Memorial Research Award. Thanks to CSIRO Australia for pHELLSGATE plasmids, Dr. Nam Hai Chua, Rockefeller University, for pER8 plasmid, Arabidopsis Biological Research Center for T-DNA insertion mutants. I will also like to extend my special thanks to my wife Opeyemi Adetola for her love and support and to all my family and friends both in the USA and back home for supporting me throughout my stay at ETSU.

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CHAPTER 1

INTRODUCTION

Control of plant diseases is very important to the agriculture of the 21st century because of the steady increase in the world's population (Alzadi et al. 2015). Different methods have been used to control plant diseases in the past although the principal and basic theme in plant control is similar in all organisms which include immunization, avoidance, exclusion, protection and immunization (Maloy 2005). The overall control of plant infection/infestation using pesticides has raised serious concerns about the food safety, environmental quality and pesticide resistance which have necessitated the need for an alternative pest/disease management system (Dordas 2009). Biological control has also been used and they offer a more environmentally friendly and safe alternative for controlling plant diseases but it has not been very effectively deployed on the fields (Emmert and Handelsman 1999). The most common practices of controlling plant diseases largely focus on genetic resistance of the host plants to pathogens and the effective management of plant and its environment. There is, however, a need for a new and effective solution to plant disease problems that provide effective control while minimizing negative consequences for human health and environment (Emmert and Handelsman 1999).

It is, therefore, important to understand the mechanisms by which plants defend themselves from various biotic and abiotic stress in order to offer a possible solution to the rising concerns on food security globally.

The Plant Immune System

For plants to grow and reproduce, they must be able to overcome colonization by many plant microbes which are detrimental to their growth and survival. Plants generally use their

innate immune system to resist pathogens (Jones and Dangl 2006) which includes massive transcription of defense genes (Eulgem 2005). The first phase which is usually very quick and it is efficient in detecting microbial invaders is achieved indirectly by sensing wounds and injury related structures in the plants that signals danger (Matzinger 2002) or directly by detecting pathogen-associated molecular pattern (PAMPs) (Jones and Dangl 2006) such as flagellin and elicitors (Zipfel and Felix 2005). This process leads to the development of a PAMP-triggered immunity (PTI) (Jones and Dangl 2006). Through evolution, some pathogens have been able to suppress the PTI by producing several effector proteins (Block et al. 2008; Göhre and Robatzek 2008) which lead to effector-triggered susceptibility (ETS) (Jones and Dangl 2006). Plants have evolved to protect themselves by recognizing these effectors by deploying the proteins like nucleotide binding-leucine rich repeat (NB-LRR) proteins resulting in the second phase of plant immunity known as the effector-triggered immunity (Jones and Dangl 2006). Ultimately, the final outcome, whether the plants are susceptible to pathogens dependent on a balance between the ability of plants to recognize and mount an effective response to the pathogen or the ability of the pathogen to suppress the host defense systems (Pieterse et al. 2009).

Studies have revealed that both PTI and ETI stimulate various pathways in plants. Besides local defense signaling, ETI and PTI activate various long distance defense reactions such as systemic acquired resistance (SAR) and salicylic acid biosynthesis (Durrant and Dong 2004), basal resistance (Jones and Dangl 2006), ethylene biosynthesis (Felix et al. 1999). Different pathways have been found to interact to bring about resistance against pathogens but not much is known about how PTI and ETI interact to bring about plant resistance. Recently, studies revealed that both PTI and ETI receptors reside in the same protein complex which makes it possible for the possible interaction of ETI and PTI signals at the very beginning (Qi et

al. 2011). However, global gene expression studies reveal that there is a temporal/quantitative difference between various players in plant immune system like PTI, ETI, basal resistance, or SAR rather than being qualitative (Katagiri 2004). This is highly suggestive of the fact that many pathogens trigger an interconnecting/common plant signaling network (Eulgem and Somssich 2007). The importance of phytohormones such as salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA) and ethylene as crucial primary signals in local as well as systemic acquired resistance has been well documented (Van loon et al. 2006; Loake and Grant 2007). Changes in the hormone levels are induced by a variety of biotic and abiotic stress condition and they mediate a variety of adaptive plant response (Pieterse et al. 2009).

Abiotic Stresses

Plant response to different stresses is a very complex process and it involves changes from the transcriptome, cellular, and physiological levels (Atkinson and Urwin 2012). Recent studies have shown that plants respond differently to multiple stresses as compared to the way they would respond to individual stress, activating a specific cascade of gene expression which relates to the exact environmental conditions encountered (Atkinson and Urwin 2012; Kumar et al. 2014). Abiotic stresses are majorly controlled by the hormone abscisic acid (ABA) (Figure 1), while defense against different biotic stresses are controlled by an antagonistic mechanism between the salicylic acid (SA), jasmonic acid (JA) and ethylene signaling pathways creating a complex network of interacting pathways (Atkinson and Urwin 2012). During vegetative growth of plants, conditions of water stress lead to an increase in the endogenous levels of ABA which helps to prevent water loss through transpiration by controlling the stomata aperture (Atkinson and Urwin 2012). ABA is also very crucial in triggering how plants respond to other adverse environmental stimuli (Zeevaart and Creelman 1988). The role of ABA in signaling abiotic stress

conditions using molecular approach have been extensively documented and it has been established that ABA-dependent mutants are affected in the regulation of various genes by cold, salt and drought (Zhu 2002).

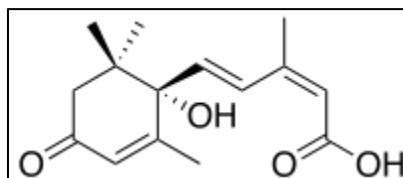


Figure 1: Chemical Structure of Absciscic Acid

The biosynthesis of ABA is regulated by osmotic stress at multiple levels. ABA-dependent and ABA-independent osmotic stress signaling first modify the vitally expressed transcriptional factors, which leads to the early expression of a transcriptional activator and this subsequently results in the activation of downstream stress tolerance gene (Zhu 2002). Salt and drought stress signal transduction consists of three pathways namely: ionic and osmotic homeostasis signaling pathways, detoxification (damage control and repair) response pathway, and pathways for growth and regulation. The ionic aspect of salt stress is signaled via the *SOS* (*salt overly sensitive*) genes (Zhu 2000) where a calcium-responsive SOS3-SOS2 protein kinase complex controls the expression and activity of ion transporters such as SOS1 (Zhu 2002). Osmotic stress then activates various protein kinases and phospholipid systems, generating an array of messenger molecules some which may function upstream of the activated osmotic stress protein kinase (Zhu 2002).

There is also cross-talks between the different plant hormones (SA, ABA, and JA or ethylene). Studies have shown that ABA is a negative regulator of the salicylic acid-dependent defense transcript *PRI* (Asselbergh et al. 2007), represses the systemic acquired resistance

(SAR) (Mohr and Cahill 2007) and prevents the accumulation of crucial defense compounds such as lignin and phenylpropanoids (Yasuda et al. 2008). Studies have also shown that SA can interfere with abiotic stress (Nemeth et al. 2002).

Biotic Stresses

Plant response to biotic stress starts from the point of recognizing pathogen-derived/ insect-derived proteins by activating some receptors, and this response leads to the synthesis and transport of three major defense hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Howe and Jander 2008; Spoel and Dong 2008). While there are many other signaling molecules like calcium, which participate in defense signaling, these three hormones are the integral part of a coordinated defense against pathogens (Bilgin et al. 2010). SA helps mediate defense response to biotrophic pathogens while JA and ET generally help mediate defense against necrotrophic pathogens (Koornneef and Pieterse 2008).

JA (figure 2A) and its methyl ester, methyl jasmonate (MeJA) (Figure 2B) are plant lipid derivatives that resemble mammalian eicosanoids in structure and biosynthesis. Jasmonic acid is distributed throughout higher plants and they are synthesized from linolenic acid via the octadecanoic pathway. Even though JAs are involved in diverse processes such as stomatal opening, root growth, tuber formation, fruit ripening and leaf senescence, they play very important roles plant defense against insects and microbial pathogens (Bari and Jones 2009). JA acts as a ‘‘master switch’’ responsible for turning on a cascade of signals in response to pathogen attack (Wasternack and Parthier 1997). Studies involving mutants affected in JA transduction reveals that there are three main components of JA signaling: coronatine insensitive 1 (COI1), jasmonate resistant 1 (JAR1) and jasmonate insensitive 1/MYC2 (JIN1/MYC2) (Bari and Jones 2009). The JIN1/MYC encodes a transcriptional factor that regulates some JA-

responsive gene expression (Lorenzo et al. 2004), JAR1 encodes an enzyme called JA amino synthase that helps in the conjugation of isoleucine to JA; a bioactive molecule perceived by plants (Staswick and Tiriyaki 2004; Thines et al. 2007) and COI encodes an F-box protein that is required for JA-mediated responses (Xie et al. 1998). With the recent discovery of jasmonate ZIM-domain (JAZ), our understanding of the molecular mechanism of JA signaling has been improving (Bari and Jones 2009). Studies have shown that COI1 complex acts as a receptor for JA-ile in *Arabidopsis* (Katsir et al. 2008). Studies have also revealed that JAZ protein acts as repressors of JA signaling which have been found (JAZ1 and JAZ3) to interact with JIN1/MYC2 and inhibit the expression of JA-responsive genes (Bari and Jones 2009). JA signaling has also been implicated in the long-distance signaling leading to systemic immunity in *Arabidopsis* (Truman et al. 2007). It is, however, important to note that the mobile signal in SAR is not JA itself but most likely to be the jasmonates (Chaturvedi et al. 2008).

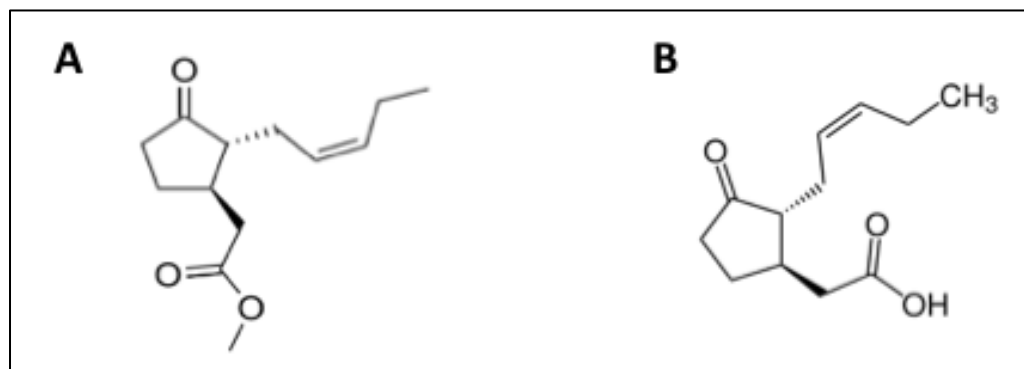


Figure 2: Chemical Structure of Jasmonates. A. Jasmonic Acid B. Methyl Jasmonate

Ethylene (Figure 3) is an important modulator in the mechanism by which plants respond to pathogens. Ethylene biosynthesis is basically regulated by environmental and endogenous signals through the differential suppression of ACC synthase genes (Bleecker 2000; Lorenzo 2004). Induced ethylene biosynthesis leads to activation of a signal transduction pathway which

consists of primary EIN3-like (Ethylene Insensitive 3) regulators and downstream ERF-like transcriptional factors (Broekaert et al. 2006). The ERF-like factor controls the expression of various effector genes involved in various aspect of plants ability to induce a systemic defense response (Broekaert et al. 2006). Studies have shown that there is a synergistic relationship between JA- and ET-signaling pathways to induce the effector genes of defense response (Ellis and Turner 2001). There are also reports that there is a significant cross-talk between the ET signaling and SA. This cross-talk which is majorly controlled by SA and jasmonate brings about a differentiated disease response.

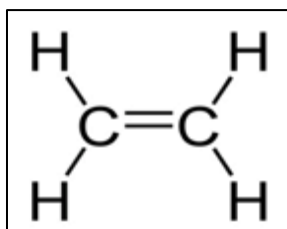


Figure 3: Chemical Structure of Plant Hormone Ethylene

Salicylic acid (SA) (figure 4A) is a natural phenolic compound present in plants which induces the production of pathogen-related (PR) proteins. When plants are infected by a pathogen, the endogenous level of SA increases (Malamy et al. 1990). This is made possible by *de novo* synthesis of SA via the isochorismate pathway and/or the phenylpropanoid pathway (Halim et al. 2006). After synthesis, SA is subjected to various modifications such as amino acid conjugation, methylation, and glycosylation (Loake and Grant 2007; Kumar et al. 2015). All these modifications lead to an effective functional use of SA in plants. For instance, glycosylation inactivates SA but allows a high amount of SA to be stored in the vacuoles. Methylation of SA is very important for long distance signaling during pathogen infections and it also plays a role in systemic acquired resistance of plants (as well as signaling innate immunity

in plants (Kumar and Klessig 2003; Dempsey et al. 2011). Methylated SA (MeSA) (Figure 4B) is a volatile ester that is normally not present in plants but is surprisingly induced when plants are infected with a pathogen (Huang et al. 2003). MeSA is synthesized by SA carboxyl methyltransferase (SAMT) (Loake and Grant 2007). A study has shown that *Arabidopsis* plants that are overexpressing *Oryza sativa OsBSMT1* accumulated MeSA and MeBA (methyl benzoic acid) and infecting this mutant with *Pseudomonas syringae* resulted in a reduced accumulation of SA, the inactive glycoside (SAG) (Koo et al. 2007). It is important to note that MeSA alone is ineffective in inducing a defense response but can function as a volatile signaling molecule during infections by pathogens (Loake and Grant 2007; Park et al. 2007). In tobacco plants, conversion of MeSA to SA acid appears to be catalyzed by a methylsterase enzyme with high affinity for salicylic acid known as SABP2 (salicylic acid binding protein 2). SABP2-silenced tobacco plants were compromised in inducing both local and systemic acquired resistance (Kumar and Klessig 2003).

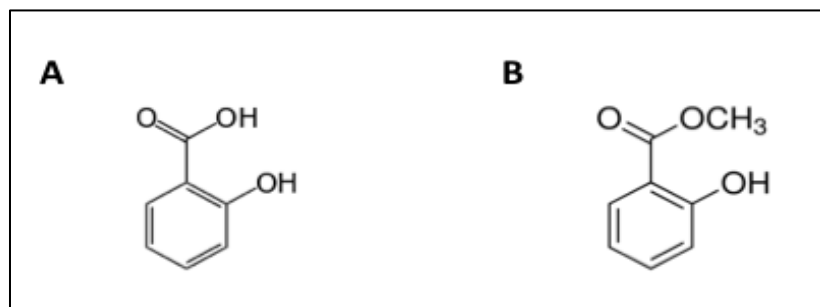


Figure 4: Chemical Structure of Salicylates. A. Salicylic Acid B. Methyl Salicylate

Salicylic Acid Binding Protein (SABP2)

In an attempt to identify the full function of how SA function in plants, several putative effectors were identified which including catalase (Chen et al. 1993), ascorbate peroxidases (Chen et al. 1993) and carbonic anhydrase which all binds to SA with relatively low affinity

(Durner and Klessig 1995; Slaymaker et al. 2002; Kumar and Klessig 2003). Recently, several other SA-binding proteins have been identified through high-throughput screens, which are glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and high mobility group box 1 (HMGB1) (Klessig 2016). SABP2 (figure 5) is a very low abundance (10 fmol/mg) soluble protein of 29 kDa (Kilo Daltons) that exhibit a high affinity for SA ($K_D=90$ nM). The binding to SABP2 to SA is generally reversible and specific to SA and its SAR inducing analogs (Kumar and Klessig 2003). Crystal structures of SABP2 confirmed that it belongs to the α/β hydrolase superfamily of enzymes (Forouhar et al. 2005). Being a member of the α/β hydrolase superfamily, the active site of SABP2 (figure 5) is defined by the presence of a catalytic triad, Ser-81, His-238, and Asp-210 (Forouhar et al. 2005). A study showed that SABP2 silenced plant in tobacco disrupted the local as well as the SAR against tobacco mosaic virus (Kumar and Klessig 2003; Park et al. 2007).

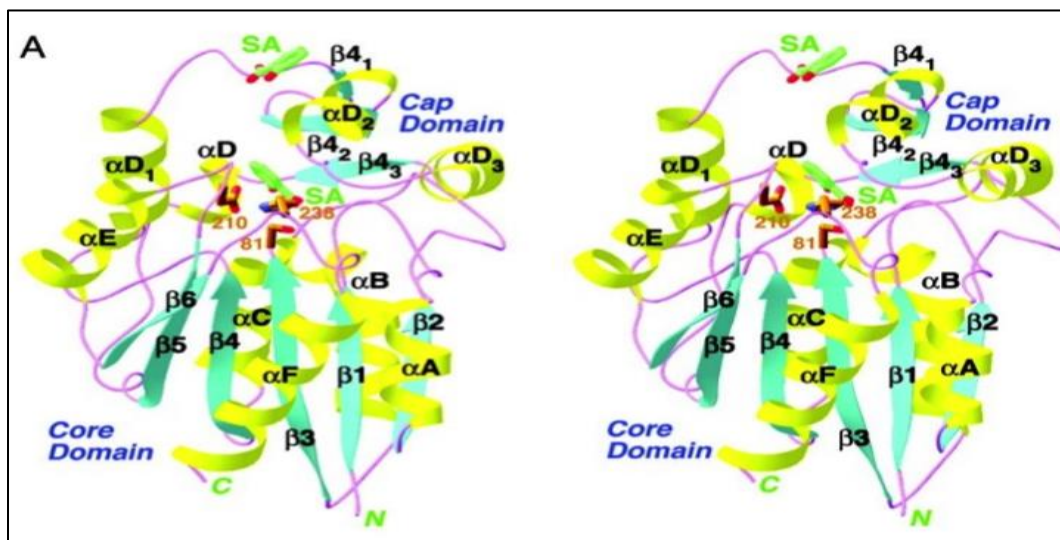


Figure 5: Structure of SABP2 in complex with SA. This figure shows the stereo view of SABP2 monomer in complex with SA. The core and the CAP domains are labeled. The secondary structure elements, α helices, β strands and loops are colored in yellow, cyan and magenta respectively. SA (in green) is located in the active site as well as another site on the surface of the enzyme (Adopted from Forouhar et al. 2005).

SABP2 Interacting Proteins (SIP)

In order to learn more about the role of SABP2 in protecting plants against pathogens through its role in SAR, a yeast two-hybrid screening was performed using SABP2 as a bait and tobacco leaf proteins as preys. It was discovered that SABP2 interacts with several tobacco leaf proteins such as SIP470 (SABP2-Interacting Protein 470) (Figure 6) (Kumar et al. unpublished). Nucleotide analysis of *SIP470* reveals that it belongs to a non-specific lipid transfer protein type 1 (nsLTP1) subfamily (Chapagai 2014).

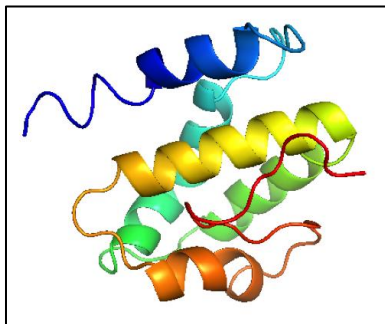


Figure 6: Predicted protein structure of SIP470. SIP470 has 5 alpha helices.

SA, SABP2 and SAR

As mentioned earlier, SA can be synthesized from both phenylalanine and chorismate pathways but the major pathway for *de novo* synthesis during an active pathogen infection is through the chorismate pathway that is catalyzed by the isochorismate synthase (ICS) (Wildermuth et al. 2001). Arabidopsis mutants lacking ICS are compromised in SAR when challenged with pathogens (Wildermuth et al. 2001). Studies suggest that an alternative and rapid way to generate SA is through the activity of SABP2 (Grant and Lamb 2006). Since SABP2 converts MeSA back to SA *in vitro* (Figure 7), this suggests that SABP2 might generate SA from signal-competent MeSA during SAR (Kumar et al. 2006).

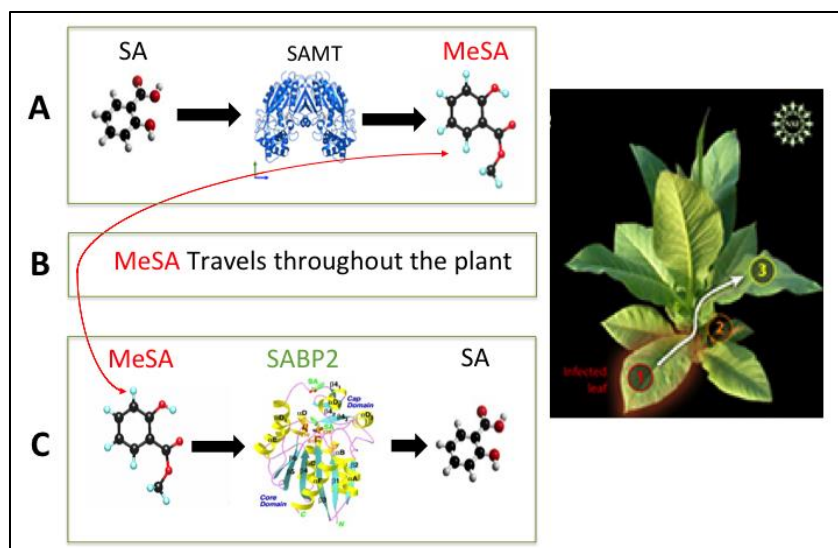


Figure 7: Plants systemic immune response. Development of SAR. SABP2 is an important component in SAR signal (Redrawn from Klessig: <http://bti.cornell.edu/>)

Lipids as Signals in SAR

Pathogen and host lipids, as well as lipid metabolites, have very important roles to play in pathogenesis as well as in the expression of defense genes in plants (Shah 2005). Lipid signals as well as Nitric Oxide (NO), SA, JA, and ethylene have all been implicated in SAR signaling (Grant and Lamb 2006). A possible role of lipid signaling in SAR is seen in a study in which mutants of the *Arabidopsis* stearoyl-acyl carrier protein desaturase (SSI2) suppressor of fatty acid desaturase 1 (*sfd1*) were compromised of SAR and had defective growth phenotype (Nandi et al. 2003). SFD1 encodes for an enzyme called dihydroxyacetone phosphate reductase that helps in forming the glycerol-3-phosphate backbone in glycerolipids (Grant and Lamb 2006). Even though SFD1 is required for the accumulation of SA locally and in distal parts of the plants, *sfd1* mutant plants do not show compromise in basal resistance which suggests that they may be involved in the generation and translocation of SAR signals (Grant and Lamb 2006). This suggests the importance of lipid a signal transfer protein in plants defense. Plant lipid

transfer proteins (LTPs) are potential candidates that are involved in transporting these lipid signal molecules.

Plant Lipid Transfer Proteins (PLTP)

Due to the hydrophobic nature of lipids or lipid derivatives involved in long-distance signaling of plants, they have to be transported by some soluble macromolecule structure (Moreau 1998). Lipid-protein complexes are an important component in many metabolic pathways but they can still function in different manners in cell signaling (Blein et al. 2002). An attempt to describe the role of these water-soluble complex in plants led to the discovery of non-specific lipid transfer proteins (nsLTPs). Lipid transfer proteins (LTPs) are a group of small, basic, conserved, 9kDa proteins that are found in high amounts in higher plants (Kader 1996). LTPs have been characterized by their ability to bind to fatty acids in-vitro and in tier ability to transfer phospholipids between membranes (Garcia-Olmedo 1995). LTPs have been classified into two major families based on their biochemical properties. Despite the fact that both families of LTPs have several similarities like having a very high PI (approximately 9), conserved 8 cysteine residues and a structure which consist of a hydrophobic cavity that is enclosed by four alpha helices, they differ generally in having a low sequence similarity and different molecular weight ranging from 9 kDa in family 1 LTP to 7 kDa in family 2 LTP (Yeats and Rose 2008).

Family 1 lipid transfer proteins generally have three amphiphilic alpha helices that are parallel to the hydrophobic lipid binding pocket (Yeats and Rose 2008). In addition to the 8 conserved cysteine residues that is present in all LTPs, the family 1 LTPs have small hydrophobic amino acids such as Ile, Val, Leu and Ala that are predominant in the LTP sequence and functions in defining the hydrophobic binding tunnel (Yeats and Rose 2008). Family 1 LTP have a semi-conserved Tyr 79 that is responsible for an enhanced intrinsic fluorescence upon

lipid binding experimentally (Lullien-pellerin et al. 1999). Studies also show that this residue is responsible for forming hydrogen bonds with the polar heads of lipids (Charvolin et al. 1999; TassinMoindrot et al. 2000). Studies using family 1 LTP isolated from rice (*Oryza sativa*) and wheat (*Triticum aestivum*) reveals that it binds to palmitic acid and L- α -phosphatidylcholine (Harvolin et al. 1999; Cheng et al. 2004).

Family 2 lipid transfer proteins have four- α helix that is bound by four disulfide bonds that enclose a hydrophobic cavity that binds to lipids (Yeats and Rose 2008). One major difference between family 1 LTPs and family 2 LTPs is the nature of pairing between cysteines 5 and 6 which makes the hydrophobic cavity of Family 2 LTPs smaller but more flexible, allowing for a variety of lipids including sterols to bind (Hoh et al. 2005).

LTPs have been implicated in number biological roles like cell signaling, antimicrobial defense, cutin synthesis, and cell wall loosening but these functions have not been well correlated with their in-vitro activity or structure (Yeats and Rose 2008).

Role of LTPs in Plants Defense

In an attempt to identify novel proteins and genes responsible defense of plants against pathogens, the antimicrobial property of LTPs was discovered (Garcia-Olmedo et al. 1995). *In vitro* screening of protein extracts from barley, Arabidopsis, spinach and onion suggested that it has a direct effect against fungal and bacterial pathogens (Cammue et al. 1995; Molina et al. 1993; Segura et al. 1993). The mechanism of action of LTP that brings about its toxicity is not well understood, but it is most likely linked to the fact that LTPs promote membrane permeability in pathogens but not in the host cells (Regente et al. 2005). Analysis of the expression pattern of LTPs revealed that most isoforms are expressed upon pathogen challenge; this gave the first support for a defensive role for this group of proteins (Van Loon and Van

Strien 1999). Another evidence of the role of LTPs on defense has been using transgenic lines overexpressing LTPs (Yeats and Rose 2008). A study showing that a family 1 LTP from tobacco could act as a binding competitor and the strength of the binding was increased by LTP-jasmonic acid complex suggests an evidence for its role in defense signaling (Buhot et al. 2004).

Role of LTPs in Plant Growth and Development

LTPs are also implicated in vegetative growth and development of plants (Yeats and Rose 2008). Studies have shown that a family 1 LTP from lily styles along with pectin polysaccharides is necessary for pollen tube adhesion (Park et al. 2000; Park and Lord 2003). Family 1 LTP has also been shown to have an important role in cell expansion and growth by promoting cell wall loosening, but the mechanism of action is not well understood but it most likely depends on the presence of a hydrophobic cavity on LTPs (Nieuwlan et al. 2009). LTPs are also indicated in cutin synthesis by acting as a carrier of a lipid precursor required for cutin formation. While there is no direct evidence to support this, studies have shown the LTP 1 are expressed in the epidermis (Suh et al. 2005) and an increase in LTP expressing leads to the accumulation of wax (Hollenbach et al. 1997) supports the hypothesis that LTPs are involved in cutin synthesis.

Role of LTPs in the Adaptation of Plants to Various Environmental Conditions

LTPs could also play a role in helping the plant survive adverse conditions. Genes induced by cold treatment have been found to code for LTP-like proteins (Hughes et al. 1992). Other LTP genes have been found to respond to salt and drought stress (Kader 1996). Barley plants were found to express LTP gene in the roots and stems during drought stress (White et al. 1994). A gene encoding for LTP-like protein in tomato is expressed in the stem when treated

with NaCl, mannitol, high temperature or ABA (Hughes et al. 1992). Just recently, a study involving overexpressed LTP 3 in *Arabidopsis* showed that the mutant had an increased tolerance to cold and drought (Guo et al. 2013).

Arabidopsis LTP12

LTP12 are members of the PR-14 family that are highly expressed in inflorescences while their expression in seedlings and leaves of plants are relatively low (Huang 2013). Microarray study on the *Arabidopsis* genome transcript expression revealed that *LTP12* are induced upon pathogen challenge (Kilian et al. 2007). *Arabidopsis* mutant *ltp12* with a seed stock number CS736658 was used in this research. It has a T-DNA insertion in the intron of the *LTP12* gene of wild-type *Arabidopsis* (Colombia-0). Binary vector (pAC161) that contains the sulfadiazine open reading frame was transformed into the plant by *Agrobacterium*-mediated transformation. The mutant is selected in the greenhouse by a sulfadiazine resistant marker (Rosso et al. 2013; Chapagai 2014). Not much is known about this mutant so far.

Arabidopsis LTP2

Promoter analysis of *LTP2* shows the presence of a box 3 and 10 base pair sequences TCATCTTCTT (Thoma et al. 1994). Box 3 is generally conserved in promoters of *Arabidopsis* phenylalanine ammonia lyase (PAL) and bean chalcone synthase (CHS) genes. PAL is involved in phenylpropanoid pathway which is found to be involved in defense (Bagal et al. 2012) while the CHS is involved in the accumulation of a stress metabolite called isoflavonoid phytoalexins and is also involved in the SA-mediated defense pathway (Dao et al. 2011). *LTP2* is highly expressed in developing seeds, leaves and in flowering plants (Arondel et al. 2000). It also shows some expression in the epidermis and sub-epidermis of the flower and embryo organs (Clark and

Bohnert 1999). Microarray study on the Arabidopsis genome transcript expression revealed that *LTP2* are also induced upon pathogen challenge (Kilian et al. 2007). Arabidopsis *ltp2* mutant with a stock number CS736752 was used in this research. It has a T-DNA insertion in the intron of the LTP 2 gene of the wild-type Arabidopsis (Columbia-0). Binary vector (pAC161) that contains the sulfadiazine open reading frame was transformed into the plant by Agrobacterium-mediated transformation. The mutant is selected in the greenhouse by a sulfadiazine resistant marker (Rosso et al. 2013; Chapagai 2014). Not much is known about this mutant so far.

Previous Studies on SIP470

Gene Expression of SIP470 upon Pathogen Infection

The changes in expression of SIP470 in tobacco plants infected with various plant pathogens, for example, TMV, *Pseudomonas syringae* pv. *tabaci* (*P.s. tabaci*) (a host-pathogen), *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and *Pseudomonas syringae* pv. *phaseolicola* (*P.s. pv. phaseolicola*) was carried out (Simo and Kumar unpublished). Results revealed that SIP470 is induced upon exposure to both host and nonhost pathogens (Simo and Kumar unpublished).

Growth Phenotype Analysis of *ltp12* and *ltp2* Mutants

In order to study the putative role of SIP470 in plants, the closest homologs of SIP470 in Arabidopsis, T-DNA insertion mutants LTP12, and LTP2 were used. Both Arabidopsis LTP2 and LTP12 show 53% and 46% similarity to SIP470 respectively (Chapagai 2014). Growth phenotype analysis of these mutants revealed that both mutants had a defective growth phenotype when compared to the wild-type (Col-0) with *ltp12* having the most significant

defective in height, the number of leaves, bolting time, inflorescence emergence time and silique formation time (Chapagai 2014).

Cloning, Expression, Purification and Lipid Binding Assay of Recombinant SIP470

SIP470 without its signal peptide was successfully cloned using gateway cloning system and expressed in *E. coli*. Recombinant SIP470 (recSIP470) was then purified using Ni-NTA affinity chromatography and ion exchange chromatography (Q-sepharose column) (Chapagai 2014). Purified recSIP470 was then used to assay for lipid binding and transfer activity using the PIP Strip membrane and the PLTP transfer assay kit respectively. Results showed that recSIP470 failed to transfer or bind to lipids under that experimental conditions (Chapagai 2004).

Hypothesis

Hypothesis 1: Tobacco SIP470 has a role in the stress signaling of plants (biotic and abiotic Stress)

Based on the preliminary findings it is hypothesized that tobacco SIP470 has an important role to play during the biotic and abiotic stress of plants. The role of various lipid transfer protein in biotic and abiotic stress has been well studied. The first evidence of the role of LTPs in biotic stress was observed in the *in vitro* screening of protein extracts from barley, *Arabidopsis*, spinach, and onion. Results showed that the extracts from these plants had a direct effect on the growth of fungal and bacterial pathogens (Cammue et al. 1995; Molina et al. 1993; Segura et al. 1993). Several LTPs have also been implicated in abiotic stress signaling. *Arabidopsis* LTP3, when overexpressed, showed an increased tolerance to cold and drought in (Guo et al. 2013). Despite the evidence on the role LTPs in both biotic and abiotic stress

signaling, their mode of action in-vivo is still widely unknown. In order to study the functional role of SIP470 in tobacco plants, the following approach will be followed:

1. Generating transgenic SIP470 tobacco plant using the RNA interference (RNAi) and Overexpression of SIP470 using an inducible plant expression system.
2. Assess the Systemic Acquired Resistance (SAR) and Basal resistance response in SIP470 transgenic plants.
3. Study the resistance (both the basal and SAR) response and defense gene expression in SIP470 transgenic plants as well as the Arabidopsis mutant plant upon exposure to biotic (pathogen), and abiotic (drought and salinity) stress.

Hypothesis 2: Tobacco SIP470 is a Lipid Transfer Protein

Based on the nucleotide analysis of SIP470 sequences obtained from yeast-2-hybrid screening that predicted that SIP470 belongs to a non-specific lipid-transfer protein 1 (nsLTP1) subfamily (Chapagai 2014), we are hypothesizing the SIP470 binds and transfers lipids in-vivo.

CHAPTER 2

MATERIALS AND METHODS

Plant Material

Two mutant *Arabidopsis* mutant lines, *ltp12* and *ltp2* in the Col-0 background were used for this study. The wild-type (Col-0) was used as a control. Various tobacco plants, a wild-type *Nicotiana tabacum* cv. Xanthi-nc NN (XNN) (Resistant to TMV), *Nicotiana tabacum* XS (Susceptible to TMV) and *Nicotiana benthamiana* were also used for this study. All *Arabidopsis* and tobacco plants were grown in a controlled environment. *Arabidopsis* seeds were sown on autoclaved soil (Fafard F-15, Agawam, MA) in a 4 x 4-inch square pots and transferred to a growth chamber (PGW 36, Conviron, Canada) set at 22°C and a 16-hour light cycle and a light intensity of about 200 $\mu\text{mol m}^{-2} \text{sec}^{-1}$. After 5 days, individual seedlings were transferred to a 4 x 4-inch square pots and allowed to grow for 3 to 4 weeks under a 9-hour light cycle at 150 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ light intensity. Tobacco seeds were similarly grown in the growth chamber set at 22°C and a 16-hour light cycle and a light intensity of about 200 $\mu\text{mol m}^{-2} \text{sec}^{-1}$. After 7-10 days, 1-2 seedlings were transferred into 4 x 4-inch square pots and maintained for 3 weeks. Plants were then transferred to single 8-inch pots (1 plant per pot) and grown for ~2 weeks before they were used for the experiments.

Chemicals and Reagents

Sodium dodecyl sulfate (SDS), β -mercaptoethanol (β -ME), tetramethylethylenediamine (TEMED), ammonium persulfate (APS), coomassie brilliant blue R-250, ponceau-S, phenylmethylsulfonyl fluoride (PMSF), magnesium chloride (MgCl_2), sodium chloride (NaCl), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), agar (Acros

organics), sucrose (Bioworld), immobilon-PSQ Membrane (0.2 μ m) (Millipore), 30% acrylamide, imidazole, methanol, ethanol, ECL western blotting substrate (Thermo Scientific), β -estradiol, Bleach, mouse monoclonal anti poly-Histidine antibody, mouse anti c-myc monoclonal antibody and Goat anti-Mouse conjugate for western (Sigma), Oligo dT-20, Taq DNA polymerase (Invitrogen, CA), Dithiothreitol (DTT), Low Molecular Weight (LMW) protein ladder (GE life Sciences), RNase free DNase (Promega), gel loading dye (Bio-Rad), QIAprep Spin Miniprep Kit, Qiagen Plasmid Midi Kit, and Gel extraction kit (Qiagen, CA), Gateway cloning kit with entry vector, pDONR221 (Invitrogen). Entry vector for overexpression (pGEM-T) was purchased from Promega. Inducible overexpression vector (pER8) was obtained from Dr. Nam Hai Chua, Rockefeller University, NY. For RNAi silencing was done using gateway based cloning where pDONR221 was the entry vector used and pHELLSGATE8 was used as the destination vector (obtained from CSIRO, Australia).

Other Materials/Instruments

SYNERGY HT multi-mode microplate reader (Biotek), Master cycler (Eppendorf, NY), spectrophotometer, Electroporator (Biorad), French press (Thermo scientific), ND-1000 Nanodrop spectrophotometer (Thermo scientific), pH meter (Beckman), LI-COR C-DIGIT Western blot imager (LI-COR), UV imaging system (UVP Bioimaging Systems), centrifuge (Beckman, model J2-21 or Sorvall RC5B), microcentrifuge (Eppendorf), Agarose (Fisher Scientific) and Polyacrylamide gel electrophoresis apparatus (Biorad), Transblot (Biorad), 1 ml syringes (BD Syringes), ultrasonicator (Fisher Scientific) and every other regular laboratory accessories (e.g. pipettes, water bath, balances) were used for this research.

Oligonucleotides (Primers)

All primers used for generating the transgenic lines were synthesized by Eurofins MWG (Huntsville, AL). Lyophilized primers were re-suspended to a final concentration of 10 μ M by re-suspending and diluting it in nuclease free water. Primers used for RNAi silencing of SIP470 and its homologs as well as overexpress SIP470 are listed in the table below:

Table 1: List of primers used for this study

SN	Primer	Sequence (5'→3')	Purpose
1	DK670 (Gateway att B1 forward primer)	GGGGACAAGTTTGTACAAAAAAGCAGGCTATTGTGGCGGCGTTAAAGGT	Silencing SIP470
2	DK671 (Gateway att B2 reverse primer)	GGGGACCACTTTGTACAAGAAAGCTGGGTATGCAGTCTTGCGGTCAGC	Silencing SIP470
3	DK685 (Gateway att B1-24 forward primer)	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGACTGCATGCACTTGCCTG	Homolog silencing of SIP470
4	DK686 (Gateway att B2-24 reverse primer)	GGGGACCACTTTGTACAAGAAAGCTGGGTACTGGACCTTGGAGCAGTCA	Homolog silencing of SIP470
5	DK674(Forward primer with Avr II site)	CCTAGGTATGGAAATGGTTGGCAAG	Overexpressing SIP470
6	DK675 (Reverse primer with Spe I site)	ACTAGTTCACCTGGACCCTGGAGCA	Overexpressing SIP470
7	DK672 (Forward primer)	GGCCCTCTAGGGAGGTGT	Screening/detection
8	DK673(Reverse primer)	TCACTGGACCCTGGAGCA	Screening/detection

Role of SIP470 in Plant Stress Signaling

Generating Transgenic Tobacco Lines Silenced in SIP470 Expression

Transgenic lines silenced in SIP470/ homologs expression were generated via RNA interference (RNAi). RNAi is a posttranscriptional gene silencing that involves the degradation of mRNA (Montgomery et al. 1998). The RNAi pathway is exploited in molecular biology to study the functions of genes (Bertile 2006). SABP2 was successfully silenced in tobacco plants using RNAi (Kumar and Klessig 2003). To generate transgenic lines using RNAi, primers were designed and synthesized with attB1 and attB2 flanking sites to amplify a region of SIP470 coding sequence that is conserved in SIP470 gene (silencing fragment) and regions that are common to the homologs of SIP470 (homolog silencing fragment). The template used for amplification was a previously sequenced clone, pDEST-17-SIP-470-25. The amplified fragment was then gel purified and cloned into the entry vector (pDNOR221) using BP clonase enzyme following manufacturer's instructions (Invitrogen). The reaction product was transformed into competent *E. coli* (DH5 α). The plasmid DNA was prepared and DNA sequenced. The SIP470 was then subcloned into the destination vector (pHELLSGATE 8) using LR clonase following manufacturer's instructions (Invitrogen). The destination vector containing SIP470 construct was then transformed into electrocompetent *Agrobacterium tumefaciens* LBA4404 which was then used to transform wild-type tobacco plants. Expression of the destination vector in the plants genome produces an intron-spliced hairpin RNA. Transformed cells were then selected using appropriate antibiotics that will be discussed later in the thesis.

Design of Primers Used for SIP470 Silencing

SIP470 nucleotide sequence was used in the BLAST tool of Sol genomics (<https://solgenomics.net/>) to query the *Nicotiana tabacum* database and identify nucleotide sequences conserved in SIP470 gene when compared to its homologs (see appendix C). A sequence of 60 base pair (bp) was identified and primers were designed to incorporate the attB1 and attB2 sites in primer sequence flanking the SIP470 nucleotide sequence for cloning into the entry vector. A similar approach was followed to search for conserved sequence present in SIP470 gene and its homolog, a sequence of 139 bp was identified and was used cloning (see appendix C).

PCR Amplification of SIP470 Silencing and Homolog Silencing Fragment

PCR amplification of silencing and homolog silencing fragments with the attB1 and attB2 flanking sides was done using PCR separately. Full-length SIP470 verified clone was used as a template (pDONR221+470 clone number P2, box 4). A 30 µl PCR reaction mixture was made which consisted 22.2 µl of sterilized water, 3 µl of PCR buffer (10 x), 0.6 µl of dNTP (50 x), and 3 µl of template (1ng-1pg / 50ml reaction), 0.6 µl of Advantage HF polymerase (50 x) and 0.6 µl of forward and reverse primer. The PCR reaction was done for 30 cycles with the denaturation temperature set at 94 °C for 30 seconds, the annealing temperature of 55 °C for 30 seconds, an extension temperature of 72 °C for 30 seconds and a final extension temperature of 72 °C for 7 minutes after 30 cycles. The amplified product was stored on ice at 4 °C and was used for further experiments.

Agarose Gel Electrophoresis

The PCR amplified product was run and detected on a 1.5 % EtBr (ethidium bromide) stained agarose gel. The gel was then used for extraction and purification of the expected fragment.

Gel Extraction and Purification

The expected amplified fragments based on the sizes for silencing and homolog silencing were excised from the gel using a clean blade and the DNA extracted using the Qiagen gel extraction kit according to manufacturer's instructions. The bound DNA from the columns were eluted using 50 µl of warm (50 °C) TE buffer and quantified using a Nanodrop.

Cloning of SIP470 Silencing Fragments into Gateway Entry Plasmid pDONR221

The gel purified SIP470 DNA was then used for cloning into pDONR221 via gateway technology following manufacturer's instructions. The reaction mixture consisted of 1 µl of the entry plasmid (pDONR221), 1 µl of BP clonase enzyme, 1 µl of the purified fragment (50 fmol) and 7 µl of TE buffer to make a final volume of 10 µl. The reaction was then incubated at 37 °C for two hours after which it was terminated by adding 1 µl proteinase K (provided in the kit). The reaction was then maintained on the ice at 4 °C and was used for transformation into competent top10 cells.

Transformation of Recombinant pDONR221-SIP470 Constructs into Top10 Cells

One µl of the product from the BP reaction (pDONR221-SIP470) was added to 200 µl of competent Top10 *E. coli* cells. The resulting mixture was incubated on ice for 30 minutes. Following incubation, the mixture was incubated for 45 seconds by placing it in a 42 °C water bath and immediately transferred to ice for 2 minutes. To the mixture 800 µl LB broth (room

temperature) was later added to the mixture and incubated in a 37°C shaker (250 rpm) for 1 hour. After one hour, transformed cells were selected by plating the mixture on an LB agar plate containing ampicillin (100 µg/ml) and incubated at 37°C overnight.

Screening of Transformed Colonies Using Colony PCR

Bacterial colonies from the plates were screened for the presence of pDONR221-*SIP470* entry clone using colony PCR. Single colonies were picked using a sterile toothpick or yellow tip, streaked on a fresh LB plate containing the appropriate antibiotics as mentioned earlier and the tip washed in 40 µl of sterile water. The tip was washed thoroughly to release traces of the bacterial colony in the water by pipetting up/down 5-10 times. Ten µl of the water was used as a template for PCR amplifications. The colony PCR mix contained of transformed bacterial cell dissolved in sterile water (10 µl), 2 µl of PCR buffer (10 x), 2 µl of dNTP, 0.2 µl of Taq DNA polymerase, 0.2 µl of M13 forward primer (vector specific), 0.2 µl of gene specific reverse primer and 4.2 µl of sterile water to make a final volume of 20 µl. The PCR reaction was done for 30 cycles in a thermocycler using the settings, 94 °C for 30 seconds, the annealing temperature of 62 °C for 30 seconds, an extension temperature of 72 °C for 80 seconds and a final extension temperature of 72 °C for 7 minutes after 30 cycles. The PCR amplified products were analyzed on 1.5% agarose gel electrophoresis as described earlier.

Isolation of Recombinant pDONR221-*SIP470* Plasmid DNA

Five positive clones for silencing and homolog silencing respectively that were verified by PCR analysis were inoculated into a 5ml LB broth containing the appropriate antibiotic and incubated in a 37 °C shaker (250 rpm) overnight. The recombinant pDONR221-*SIP470* plasmid DNA was purified using QIAprep Spin MiniPrep Kit (Qiagen) according to the manufacturers'

instruction. The resulting plasmid was quantified using a Nanodrop spectrophotometer and stored at -20 °C.

Sequencing of Recombinant Plasmid

Eight µl of the purified plasmid DNA (~800ng) was transferred to a PCR tube containing 2 µl of M13 forward primer. The mixture was sent for DNA sequencing to the DNA analysis facility at Yale University. Sanger method of sequencing was used.

Cloning of SIP470 Silencing Construct into pHELLSGATE 8

The verified pDONR221_SIP470 construct was used for subcloning SIP470 into the destination vector pHELLSGATE 8. The reaction mixture consisted of 1 µl of destination vector, 1 µl of LR clonase enzyme, 1 µl of recombinant plasmid DNA of pDONR221 and 7 µl of TE buffer (pH 8.0) to make a final volume of 10 µl reactions. The reaction was then incubated at 37 °C for two hours after which it was terminated by adding 1 µl proteinase K. The reaction was then maintained on the ice at 4 °C and was used for transformation into competent Top10 cells. One µl of the product from the LR reaction (pHELLSGATE8-SIP470) was transformed into competent *E. coli* as described earlier. The transformed cells were selected on LB agar plates containing spectinomycin (50 µg/ml) and incubated at 37 °C. Bacterial cells from the transformation were screened for the presence of pDONR221-SIP470 entry clone using colony PCR as earlier described. The PCR amplified products were analyzed on 1.5% agarose gel electrophoresis as described earlier. Five positive clones for silencing and homolog silencing respectively that were verified from the colony PCR analysis was inoculated into a 5ml fresh LB broth containing the appropriate antibiotic and incubated in a 37 °C shaker (250 rpm) overnight. The recombinant pHELLSGATE8-470 plasmid was isolated and purified using QIAprep Spin

MiniPrep Kit (Qiagen) according to the manufacturers instruction. The resulting plasmid was quantified using a Nanodrop spectrophotometer and stored at -20 °C.

Transforming *Agrobacterium tumefaciens* LBA 4404 cells with pHELLSGATE-SIP470

Silencing Construct

To revive the LBA 4404 strain of *Agrobacterium*, it was streaked from -80 °C glycerol stocks onto an LB plate containing streptomycin and spectinomycin antibiotics (100 µg/ml) and incubated for 2 days at 28 °C. A single colony was then added to 5 ml tube of LB with the appropriate antibiotics and grown overnight on a 28 °C rotating shaker. The overnight culture was then diluted (1:100) in 100ml LB and incubated at 28 °C with shaking until an OD₆₀₀ = 1.0 was reached. The cells were then centrifuged at 4000rpm for 10 minutes at 4 °C. The supernatant was then discarded and the pellet was resuspended in 5ml of ice-cold 20mM sterile calcium chloride. The cells were again centrifuged at 4000rpm for 5 minutes at 4 °C. Resulting pellets were resuspended in 1ml of ice-cold 20mM calcium chloride and 100 µl were aliquoted into 2 ml Eppendorf tubes containing 100 µl 50% glycerol and snap frozen in liquid nitrogen. For electroporation, 1 µl of plasmid DNA for silencing construct (247ng/µl) and homolog silencing construct (150 ng/µl) was added to 20 µl each of electrocompetent LBA 4404 cells respectively. This mixture was then transferred to ice chilled 0.2 cm electroporation cuvette (Biorad). The cells were electroporated using a pulse of 2.20 kV (kilovolts) for 5.4 milliseconds and 1 ml of SOC media (appendix B) was added to the electroporated cells. The cells were then transferred into a sterile Eppendorf tube and incubated at 28 °C for 3 hours. Cells (50 µl) were plated on an LB agar containing appropriate antibiotics and incubated at 28 °C for 1-2 days for colonies to develop. Transformed *Agrobacterium* colonies were screened for the presence of pHELLSGATE8-SIP470 clone using colony PCR as earlier described. Gene specific primers

were used to verify the presence of an insert of interest. Verified clones were used for plant transformations (both transient and stable).

Cloning SIP470 to Generate Overexpression Clone

To overexpress SIP470 in transgenic tobacco plants, the full-length SIP470 cDNA was cloned into pER8 plasmid. pER8 is an estradiol-inducible plant expression vector. Using an inducible system allows flexibility for studying the functions of genes whose expression is developmental or regulated to a specific tissue (Zuo et al. 2000). pER8 is an estrogen receptor-based chemical inducible system that comprises of a chimeric transcriptional activator XVE which was assembled by fusion with a DNA binding domain of the bacterial repressor LexA (X), transactivating domain and human estrogen receptor (E; ER) regulatory region. Estrogen highly regulates the transactivating activity of the chimeric XVE factor whose expression is controlled by the strong constitutive promoter G10-90 (Zuo et al. 2000).

Primers were designed to incorporate restriction sites Avr II and Spe I at the 3' and 5' end of *SIP470* gene respectively. Full-length SIP470 gene was amplified from (pDONR221+470 clone#P2). A 30 µl PCR reaction mixture was made which consisted 22.2 µl of sterile water, 3 µl of PCR buffer (10 x), 0.6 µl of dNTP (50 x), and 3 µl of template (1ng-1pg / 50ml reaction), 0.6 µl of Advantage HF polymerase (50 x) and 0.6 µl of forward and reverse primer. The PCR reaction was done for 30 cycles with the denaturation temperature set at 94 °C for 30 seconds, the annealing temperature of 55 °C for 30 seconds, an extension temperature of 72 °C for 30 seconds and a final extension temperature of 72 °C for 7 minutes after 30 cycles. The PCR amplified products were analyzed on 1.5% agarose and the SIP470 amplified DNA band was excised from the gel. The DNA was purified using the Qiagen gel extraction kit according to

manufacturer's instructions. The purified product was eluted using 50 µl of 50 °C TE buffer and quantified using a Nanodrop spectrophotometer.

Cloning SIP470 Overexpressing PCR Amplified cDNA into the pGEMT

The ligation reaction was done following the manufacturer's instruction (Promega). The ligation reaction mixture consisted of incubating pGEMT vector (50 ng/µl) with 5 µl of purified *SIP470*, 1 µl of T₄ DNA ligase (3 Weiss units/ µl) and 2 µl of buffer (1x). The mixture was incubated at 28 °C for 1 hour and the resulting product was used for further experiments. Two µl of the ligation mixture (pGEMT-SIP470) was added and mixed gently to 200 µl of chemically competent *E.coli* Top10 cells. The transformation was then done according to the method described earlier. Transformed cells were plated on LB plates containing Ampicillin (50µg/ml). Bacterial colonies were screened for the presence of SIP470 using colony PCR as earlier described earlier. The primers used were T7 forward primer (plasmid specific) and a gene specific primer. The PCR product was analyzed by agarose gel electrophoresis. Five positive colonies were used for plasmid DNA isolation as described earlier. The resulting plasmid was quantified sequenced.

Cloning of SIP470 into pER8.

Verified clone (pGEMT-SIP470-4) (210.5 ng/µl) was used for cloning into pER8. First, SIP470 was excised out of pGEMT by restriction digestion. A total 20 µl digestion mix was prepared that consisted of 10 µl pGEMT-SIP470-4, 2 µl of the NEB cutsmart buffer, 7 µl sterile water, 0.5 µl Avr II and 0.5 µl of Spe I. The mixture was incubated for 3 hours at 37 °C. Similarly, the pER8 vector was also digested. The digestion mixture consisted of 3 µl of the pER8 vector, 1 µl of cut smart buffer (2), 5 µl of sterile water, 0.5 µl Avr II and 0.5 µl of Spe I.

The mixture was incubated for 15 minutes at 37 °C and 1 µl was added to the reaction mix. Both the SIP470 fragment and the pER8 were excised out and used for gel extraction as described earlier. The purified product was eluted and quantified. For overexpressing *SIP470*, the pER8 vector and pGEMT-*SIP470* were ligated. The ligation mixture consisted of 1.5 µl of digested pER8 vector (45ng/ml), 0.5 µl of digested pGEMT-470-4 (14.4ng/ml), 3 µl of sterile distilled water and 5 µl buffer and T4-DNA ligase enzyme. The mixture was incubated at 25 °C for 1 hour. Two µl of the ligation mixture (pER8-*SIP470*) was added to 200 µl of chemically competent *E. coli* Top10 cells. The transformation was done as described earlier and the transformed cells plated on LB plates containing spectinomycin (50 µg/ml) and incubated at 37°C overnight. Bacterial colonies were screened for the presence of pER8-*SIP470* using colony PCR as earlier described. pER8 vector forward primer and the reverse primer was used for detection of expected band. The PCR product was run on a 1.5% EtBr stained agarose gel electrophoresis for verification of inserts. The recombinant pER8-SIP470-Overexpression plasmid was purified using the boiling method. Five positive colonies were inoculated into a 5 ml fresh LB broth containing the appropriate antibiotic and incubated in a 37 °C shaker (250 rpm) overnight. The overnight grown culture (1.5 ml) was then transferred to a microcentrifuge tube, and centrifuged for 60 seconds at 12,000rpm at 4°C. The pellets were resuspended in 500 µl of ice cold STE (appendix B) buffer and centrifuged 60 seconds at 12,000rpm in 4°C. Resulting pellets were again resuspended 350 µl of ice cold STET solution (appendix B) and 25 µl of lysozyme was added to the suspension and boiled for 50 seconds. After boiling, the solution was then centrifuged for 10 minutes at 14000 rpm at 4 °C. The supernatant was transferred into a new microcentrifuge tube and precipitated with equal volume of isopropanol. The solutions were mixed by inverting the tubes and centrifuged for 30 minutes at 14000 rpm at

4 °C. The supernatant was discarded and the pellet was washed using 70% ethanol by inverting the tubes and centrifuging it for 10 minutes at 14000rpm at 25°C. The resulting pellet was resuspended in 25 µl of TE buffer, quantified and DNA sequenced as described earlier.

Transformation of pER8-SIP470 to LBA 4404 Competent Cells

To transform the electro-competent LBA 4404 *Agrobacterium tumefaciens* cells, 1 µl of pER8-SIP470 plasmid DNA (142ng/ml) was added to 20 µl of cells pre-thawed on the ice. The mixture was then transferred to 0.2 cm electroporation cuvette (Biorad). The cells were electroporated and 1 ml of SOC media (Appendix B) was added to the electroporated cells. The cells were then transferred into a sterile Eppendorf tube and incubated at 28 °C for 3 hours. Fifty µl of the cells were plated on LB plates containing spectinomycin and streptomycin (50 µg/ml). The plates were incubated at 28 °C overnight.

Transient Expression of pER8-SIP470 in *Nicotiana benthamiana*

To check if the verified clone is expressing correctly upon induction, a transient expression was done in *N. benthamiana*. The verified pER8-SIP470 clone was inoculated into a 5ml LB broth containing the appropriate antibiotics and incubated in a 28 °C shaker overnight. The overnight culture was sub-cultured in a 20 ml LB with appropriate antibiotics, containing 20 µM acetosyringone (3'5'-dimethoxy-4'-hydroxyacetophenine, Aldrich) and 500 µl of 10 mM MES (pH 5.5). The culture was incubated in a 28 °C shaker overnight. The overnight grown cells were then harvested by centrifugation. The cells were resuspended in 10mM (MES) and 10mM MgCl₂. The process was repeated. The cells were then suspended in buffer containing 10mM MES pH 5.5, 10mM MgCl₂ and 150 µM acetosyringone. The concentration of cells was adjusted to OD₆₀₀=0.5 and incubated at room temperature on a shaker overnight. Next day the bacterial

suspension was infiltrated in *N. benthamiana* leaves using a needleless 1 ml syringe. The plants were returned to the growth chamber and left overnight. The next day, the infiltrated leaves were spray treated with 50 μ M β -estradiol in 0.01% tween 20 and left in the growth chamber overnight. Leaf disc samples were then collected, snap frozen in liquid nitrogen and processed for protein extraction, SDS-PAGE and western blot analysis.

SDS-PAGE

The leaf disc samples were homogenized using a mechanical homogenizer in 100 μ l of 2X SDS sample buffer containing β -Mercaptoethanol (β -ME) was added to the sample and boiled for 4 minutes. The sample was then centrifuged at 13,000 rpm for 10 minutes. The SDS gel was then run at 30 mA constant current for 1 hour in a running buffer (as Appendix B).

Western Blot Analysis

The separated proteins from the gel were transferred to PSQ membrane (0.2 μ M). The PSQ membranes were prepared by soaking it in 100% methanol for 15 seconds and immediately rinsed with distilled water twice before incubating it in 1x transfer buffer (Appendix B) for 15 minutes. The SDS gel with the PSQ membrane was then sandwiched between a sponge and 3mm Whatman filter paper. Transfer of protein from the gel to the membrane was then carried out at 94 volts for 1 hour at 4 °C in a Biorad Transblot cell. After the transfer, the membrane was soaked in methanol for 10 seconds and allowed to dry. After drying, the membrane was soaked in methanol for 10 seconds. The membrane was later stained with ponceau-S (0.1% ponceau-S and 5% acetic acid) for 2 minutes to verify the protein transfer. The ponceau-S stain was later rinsed off completely using 1xPBS (Appendix B). The blot was then incubated with the monoclonal anti-C-myc antibody (primary antibody) in 5 ml blocking buffer (1% dry milk, 3%

BSA in 1X PBS) and incubated overnight (~16h) at 4°C on a shaker. The next day, primary antibody was removed and the membrane was washed for 3 times (5 min each) using 1x PBS first, followed by 1x PBS containing 3% tween 20, and finally rinsed with 1x PBS. The membrane was then incubated with anti-mouse IgG peroxidase conjugate (1:10,000 in 5 ml of blocking buffer) at room temperature for 1 hour on a shaker. The membrane was subjected to washing as described above. Following washing, the membrane was incubated in the ECL substrate (1:1) for 5 minutes and scanned using the C-DiGiT blot scanner (LiCOR) as described by the manufacturer. The blot was later stained with coomassie blue to visualize total loaded proteins.

Transformation of Tobacco with Agrobacterium LBA 4404 containing pER8-SIP470 and pHELLSGATE8-SIP470

Verified Agrobacterium clones for silencing and overexpression were grown in LB media with the appropriate antibiotics at 28 °C for 2 days to until stationary phase was reached. The culture was then diluted using an equal volume of LB and 20 ml of the diluted culture was transferred to a sterile 50 ml tube. Whole leaves from wild-type tobacco plant (XNN) was collected and surfaced sterilized by treated it with 20% (v/v) commercial bleach containing 0.1% tween-80. The leaves were later rinsed about 3 times using sterilized water to remove beach. Leaf disc was punched from the sterilized leafs using a sterile cork borer and about 50 leaf disc were incubated with the Agrobacterium cultures respectively. The bacteria cultures were left on the shaker at room temperature for 30 minutes before plating the leaf disc inverted on the shoot inducing media (SIM) (Appendix B) with no antibiotics. The plates were later incubated at 23 °C in the dark for 2 days. After incubation for 2 days, the leaf disc was incubated in 20 ml of LB media containing carbenicillin (100 µg/ml) and 3% sucrose and kept on the shaker for 2 hours.

The leaf discs were taken out, gently blotted between autoclaved filter papers and then transferred to the SIM media plates. The media contained carbenicillin (100 µg/ml) and hygromycin (20 µg/ml) for pER8 constructs and carbenicillin (100 µg/ml) and Kanamycin for pHELLSGATE 8 constructs selection. The plates were incubated at 23 °C /16hrs light until calli development which later gave rise to shoots. When the young shoots that were large enough (~1 inch tall) was transferred into an RIM (Root-inducing media) media containing antibiotics (Appendix B). Rooted plants were transferred to the soil.

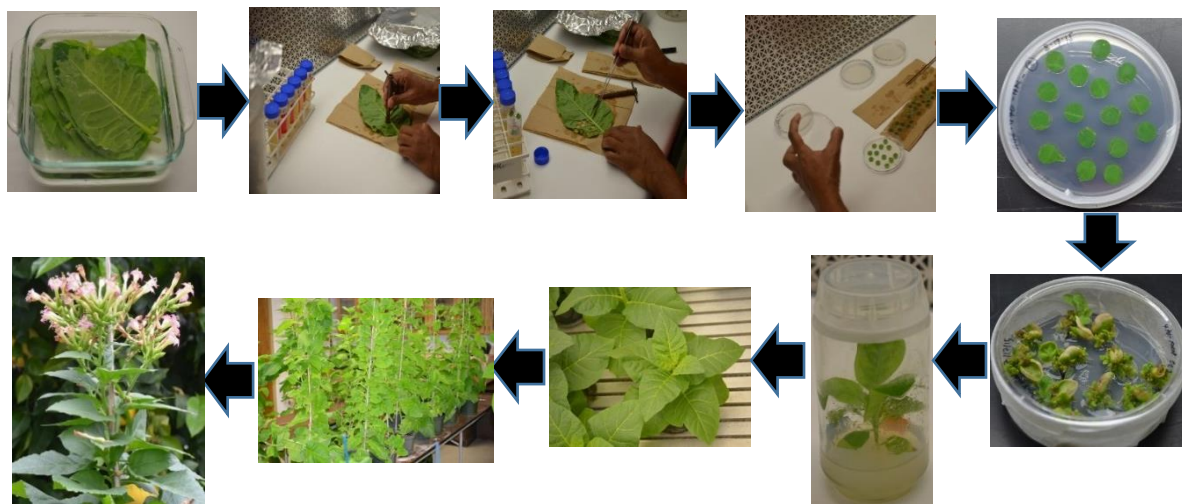


Figure 8: Various steps involved in making transgenic tobacco RNAi lines through Agrobacterium-mediated transformation

Screening of Transgenic lines for SIP470 silencing

Transgenic lines were screened for silencing and homolog silencing using RT-PCR (Reverse Transcriptase PCR). This method involved isolation of mRNA from various selected transgenic lines and checking for expression of SIP470.

Total RNA Isolation and cDNA Synthesis

A total 3 to 4 leaf discs were frozen in liquid nitrogen and homogenized to a fine powder using a blue pestle and a mechanical grinder. To the homogenized leaf powder, 0.5 ml Trizol was added and mixed by vortexing. An additional 500 μ l of trizol was added to the sample and then transferred into a 2 ml screw-cap tubes containing ~0.2ml of 2 mm autoclaved zirconia beads. Samples were immediately homogenized for 45 seconds at 4.5 power setting using FastPrep 24. To the homogenized sample, 200 μ l of chloroform was added and mixed by inverting the tubes vigorously for 15 seconds before incubating the samples at room temperature for 10 minutes. After incubation, the samples were centrifuged at 12000 x g for 15 minutes at 4 °C and the upper aqueous phase was transferred to new 1.5 ml tube. 500 μ l of isopropanol was immediately added to the tube and the tube was incubated for 10 minutes at 30 °C. Samples were then centrifuged at 12000 x g for 10 minutes at 4 °C and the supernatant was discarded. To the resulting pellet, 75% cold ethanol was added and the samples were mixed by vortexing. Samples were then centrifuged at 7500 x g for 5 minutes at 4°C and the supernatant was air dried for 10 minutes. Air dried pellets were resuspended in 40 μ l of DEPC treated water (diethylpyrocarbonate) (appendix B), vortexed few times and incubated at 55 °C for 10 min. To the fully resuspended RNA, 10 μ l of RNase free DNase-buffer mix (2 μ l DNase + 5 μ l DNase buffer + 3 μ l DEPC treated water) was added and incubated at 37 °C for 20 minutes. To remove the DNase, the sample was treated again with trizol reagent, chloroform, isopropanol, and chloroform using half the volume described above. The resulting pellet was then collected and then air dried for 10 minutes. The air dried sample were then resuspended using 20 μ l of DEPC water and incubated for 5-10 minutes at 55 °C to ensure that the pellets were dissolved. RNA

concentration (ng/ μ l) was measured using Nanodrop spectrophotometer (Nanodrop Technologies ND-1000). Samples were stored at -80°C until ready for cDNA synthesis.

First Strand cDNA Synthesis

Reverse transcriptase enzyme was used to synthesize first-strand complementary DNA (cDNA) from the isolated total RNA. A two-step process was used for this experiment. In the first step, 2 μ l of oligo-dT (0.5 μ g/ml) was added to 1 μ g of total RNA (diluted to a total volume of 8 μ l with DEPC treated water) and the mixture was incubated at 75°C for 10 min followed by immediate cooling to 4 °C in the thermocycler (Eppendorf). The samples were then cooled to 4 °C. 10 μ l mixture that consisted of 1 μ l reverse transcriptase (RT) (MMLV), 4 μ l 5X RT buffer, 1 μ l RNasin (RNAase inhibitor), 1 μ l 10 mM dNTPs mix, and 3 μ l DEPC treated water was added to the sample. The samples were then immediately vortexed and incubated for 60 minutes at 42 °C and then at 70 °C for 10 minutes in the thermocycler. The newly synthesized cDNA samples were then stored at -20 °C until further experiments.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The synthesized cDNA were used to screening for the expression of *SIP470* in the silenced and homolog silenced transgenic lines. A 10 μ l PCR reaction mixture that contained 6 μ l of DEPC water, 1 μ l 10 X dNTP, 1 μ l 10X Taq polymerase buffer, 0.2 μ l Taq DNA polymerase, 0.4 μ l of 10 μ M forward primer, 0.4 μ l of 10 μ M reverse primers and 1 μ l of cDNA sample.

Screening of SIP470 Overexpressor Transgenic lines

Overexpressor transgenic lines were screened for successful expression of SIP470 protein in the plants by inducing leaf disc samples using β - estradiol (50 μ M) and detecting the

overexpressed SIP470 using western blot and SDS-PAGE. Leaf disc samples were collected using a cork borer (#6). Each leaf disc was placed in a 24 well cell culture cluster plate containing 50 μ M β -estradiol in 0.01% tween 20 and left overnight. The samples were then homogenized in liquid nitrogen. 100 μ l of SDS buffer was added to the samples and analyzed using western blot as earlier described.

Role of SIP470 in Biotic Stress

Role of SIP470 in systemic acquired resistance (SAR)

To assess the role of SIP470 in the induction of SAR, *Arabidopsis* mutants (*ltp 12* and *ltp 2*) were used. Bacterial growth was measured in the leaves by homogenizing the leaves, diluting the extract and plating it on selective antibiotic media (LB) and counting the bacterial colonies to determine the colony forming units (cfu/ml) (Kiefer and Slusarenko 2003). SAR was investigated based on a modified protocol from Kiefer and Slusarenko (2003). To induce SAR, the two lower leaves of control (A.t. col 0) and mutant *Arabidopsis* plants were the first syringe infiltrated with avirulent pathogen (OD=0.002) (*P.S tomato DC3000 AvrRPT2*) or 10 mM MgCl_2 (control). After 2 days post infection (2 DPI), three upper leafs were challenged with a virulent pathogen (OD=0.0002) *Pseudomonas syringe pv. maculicola*. Leaf discs (5mm) were collected from the treated upper leaves using a cork borer and homogenized in 1ml distilled water using FastPrep 24 for 5 seconds at least twice. A 10-fold serial dilutions was prepared in sterile water, and an aliquot of 20 μ l was spotted on an LB agar containing rifampicin 100 μ g/ml. Plates were incubated at 28 $^{\circ}\text{C}$ until the bacterial colonies developed and were large enough to be counted. All the bacterial colonies were counted, multiplied by the dilution factor to get cfu/ml. The experiments were done in triplicates.

Role of SIP470 homologs in Basal Resistance

To assess the role of SIP470 in basal resistance, the bacteria growth was quantified from an infected leaf. *Arabidopsis* mutant and control plants were infiltrated with virulent pathogen (OD=0.0002) *Pseudomonas syringe* Pv. *maculicola*. At 3 days post inoculations (DPI), Leaf discs (5mm) were collected and processed for plating as described for SAR experiment. Experiments were done in triplicates.

Role of SIP470 Homolog in Abiotic Stress

To assess the role of SIP470 in abiotic stress, ltp12, and Col 0 seeds surfaced sterilized by incubating it in 70% ethanol for 5 minutes. The seeds were then incubated in 20% (v/v) commercial bleach containing 0.1% Tween 20. After surface sterilization, the seeds were then washed with sterile distilled water at least 4 times and plated on half strength MS media containing Gamborg vitamins. Plated seeds were then kept at 4°C for 2 days before transferring to the lighted racks. Seedlings (~5-7 days old) were then transferred to MS media plates containing various concentrations of stress-inducing chemicals (NaCl, Hydrogen Peroxide, and Absciscic Acid) (See appendix for media composition).

Gene Expression Studies

To understand at the molecular level the possible function of *Arabidopsis* LTP12, a gene expression study was conducted. *Arabidopsis* wild-type plant (Col 0) and mutant plants were infiltrated with virulent pathogen (OD=0.001) *Pseudomonas syringe* Pv. *maculicola*. 2 Leaf disc samples were collected at 0, 3, 6, 9, 12, 24 and 48 hours post-infection from mutant and wild-type plants using a cork borer of size 2. Leaf disc samples were then homogenized in liquid

nitrogen and RNA was extracted, resulting cDNA was synthesized and RT-PCR was carried out as described earlier.

Tobacco SIP470 is a Lipid Transfer Protein (Hypothesis 2)

Expression of Recombinant SIP470 in *E.coli*

The SIP470 clone, pDEST17-SIP-470-25 was used for recombinant protein expression. The chemically competent BL21 (DE3) pLysE cells (100 µl) were transformed with the pDEST17-SIP-470-25 construct and selected on antibiotics plate as described earlier. Expression of recombinant SIP470 was done according to the method described by Chapagai (2014).

In order to determine the appropriate temperature that resulted in a more soluble form of recombinant SIP470, an overnight culture from a single colony was grown in LB media containing 100 µg/ml ampicillin and 10 µg/ml kanamycin at 17 °C and 37 °C respectively. The overnight culture was diluted 100 times into fresh LB media containing the appropriate antibiotics and then incubated at 37 °C until OD₆₀₀= 0.6. Protein expression was induced by adding 10 µM IPTG (Isopropyl β-D-1-Thiogalactopyranoside (IPTG) to the bacteria culture and incubating it at 37 °C for 3 hours and 17 °C overnight respectively in a shaker. The bacterial pellet was collected by centrifugation at 13,000 rpm for 3 minutes at room temperature. The pellet was suspended in 1X Ni-NTA binding buffer (50 mM sodium phosphate monobasic, 300 mM sodium chloride, 10 mM imidazole, pH 8.0) and cells were broken by sonication with 20% amplitude for 15 seconds. The process was repeated 3 times. The supernatant (soluble) and pellet (insoluble) were separated by centrifugation at 13,000 rpm for 15 minutes in 4°C. The pellet was resuspended in 1X Ni-NTA binding buffer. Proteins from both soluble and insoluble fractions

were run on SDS gel and western blotting was performed to determine the solubility of recombinant SIP470.

Purification of Recombinant SIP470 by Ni-NTA Column Affinity Chromatography

An overnight culture from a single colony was grown at 37°C in 5 ml LB media containing 100 µg/ml ampicillin and 10 µg/ml kanamycin. Then, the overnight culture was diluted 100 times into fresh LB media containing 100 µg/ml ampicillin and 10 µg/ml kanamycin and then incubated at 37°C until the OD₆₀₀ = 0.6. The bacterial culture was induced with 10 µM IPTG as a final concentration and then incubated at 37°C overnight in a shaker. The bacterial pellet was collected by centrifugation at 8,000 rpm for 15 minutes at 4°C and 42 then resuspended in 1X Ni-NTA buffer. Protease Inhibitor (PI) (10 µl/ml) was added to the suspended bacterial pellet. Bacterial cells were lysed in a French press (Thermo Electron Corporation; cell type 20 K) and the soluble fraction (supernatant) was collected by centrifugation at 13, 000 rpm for 15 minutes at 4°C. The soluble fraction was then incubated overnight with 1x Ni-NTA resin (Qiagen) at 4°C. The unbound proteins were removed by washing with washing buffer containing 10 mM Imidazole in 1x Ni-NTA buffer. The bound recSIP470 was then eluted with elution buffer containing 250 mM Imidazole in 1x Ni-NTA buffer at room temperature. The presence of recSIP470 was confirmed by western blotting using monoclonal anti-polyHistidine antibody. SDS-PAGE analysis and Western Blot analysis was performed as described earlier. The primary antibody used was an anti-polyHistidine antibody (1:3000).

In vitro Lipid Binding Assay

In order to test if SIP470 is a lipid transfer protein, an *in vitro* lipid binding assay was performed using soluble NI-NTA purified SIP470 and TNS (*p*-toluidinonaphthalene-6-sulfonate), a fluorescent lipid binding substrate (Debono et al. 2009). TNS fluoresces intensely when bound in a hydrophobic environment but weakly in aqueous solution (Debono et al. 2009). TNS binding assay was performed from a protocol adopted from Debono et al. (2009) with slight modifications. 250 nM of recSIP470 (final concentration) and assay buffer (175 mM mannitol, 0.5 mM K₂SO₄ 0.5 mM CaCl₂, and 5 mM MES, pH 7.0) was incubated in a 96 well plate to a final assay volume of 100 µl. To the mixture, 250 µM of TNS solution was then added incrementally (1-20 µM) and fluorescence was read using a Synergy HT microplate reader at an excitation and emission wavelength of 320 and 437 nm respectively. Fluorescence was then read by subtracting the fluorescence values from the control which contained no protein but contains the elution buffer (see appendix) used for purifying the recombinant protein.

Effect of SABP2 on Lipid Binding Activity of SIP470

To investigate the possible effect of SABP2 on the binding activity of a lipid-binding assay in the presence of SABP2 was carried out. The purified recombinant SIP470 (250 nM, final concentration) and 500 nM of SABP2 were incubated together for 10 minutes. The mixture was then mixed with assay buffer (175 mM mannitol, 0.5 mM K₂SO₄, 0.5 mM CaCl₂, and 5 mM MES, pH 7.0) to a final volume of 100 µl in a microplate. To the mixture, TNS solution (250 µM) was then added incrementally (1-20 µM). The fluorescence was read using a Synergy HT microplate reader at an excitation and emission wavelength of 320 and 437 nm respectively.

CHAPTER 3

RESULTS

Role of *SIP470* in Plant Stress Signaling (Hypothesis 1)

Generating *SIP470* RNAi Silenced Transgenic Tobacco Lines

In order to investigate the possible roles of *SIP470* in biotic as well as abiotic plant stress signaling, there was a need to generate transgenic lines in tobacco that are silenced in the expression of *SIP470*. Similar, we speculated that there was also a need to equally silence the homologs of *SIP470* in order to avoid a functional complementation using one of *SIP470* homologs in silenced plants. Transgenic lines that are silenced in *SIP470* were generated as explained in the methods section. A gateway based plasmid, pDONRR221 was used to generate an entry clone which facilitated recombination with the destination vector pHELLSGATE 8 (Figure 9). The destination vector was transformed into competent *Agrobacterium tumefaciens* LBA4404 cells which were used for the plant transformation.

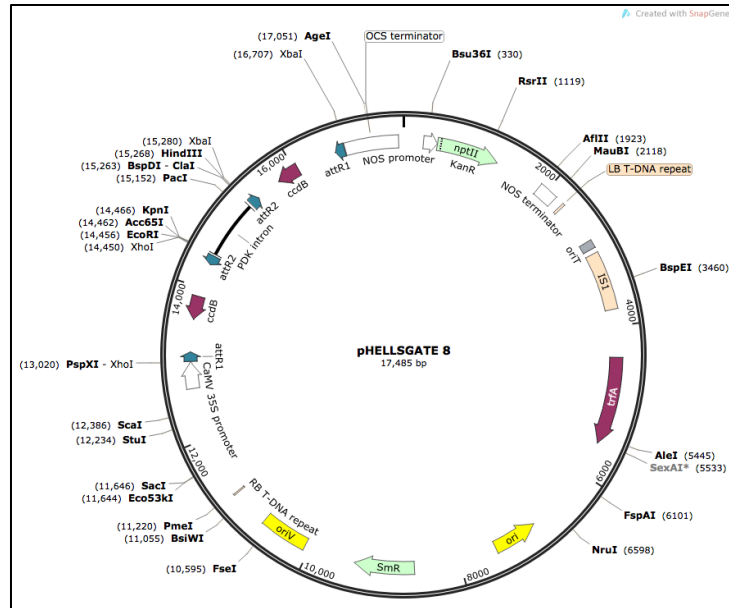


Figure 9: Vector Map of pHELLSGATE 8 used in generating SIP470 transgenic lines.

PCR Amplification of SIP470 Silencing Fragment

To generate silencing and homolog silencing construct, selected fragments in SIP470 coding sequences for silencing (Figure 10) and homolog silencing (Figure 11) was amplified from a verified template (pDONR221+470 clone number P2) using a primer that has flanking attB1 and attB2 flanking sites. PCR was then performed as earlier described in the methods section. Results show a successful amplification of silencing fragment (Figure 12A) and homolog silencing fragment (figure 12B). The resulting fragments were used for subsequent clonings.

ATGGAAATGGTTGGCAAGATTGCGTGCTTTGTGTTACTTTGCATGGTGGTGGTTGCACCCCA
TGCAGAGGCACTGAGCTGCGGCCAGGTTTCAGTCTGGCCTGGCTCTTTGCCTCCCTTATCTGC
AGAGTCGCGGCCCTCTAGGGAGGTGTT**TGTGGCGGCGTTAAAGGTGTGTTGGGTGCTGCCCCG**
ACCCAGCTGACCGCAAGACTGCATGCACTTGCCTGAAATCAGCTGCTTATGCTATTAAGGG
TATTAATATGGGCAAAGCCGCTGGACTTCCTAGTGCTTGTGGCGTTAACATTCCCTACAAGA
TCAGTCCCTCTACTGACTGCTCCAGGGTCCAGTGA

Figure 10: Verified Sequence of SIP470 showing 60bp long sequence selected for silencing (underlined).

ATGGAAATGGTTGGCAAGATTGCGTGCTTTGTGTTACTTTGCATGGTGGTGGTTGCACCCCAT
GCAGAGGCACTGAGCTGCGGCCAGGTTTCAGTCTGGCCTGGCTCTTTGCCTCCCTTATCTGCAG
AGTCGCGGCCCTCTAGGGAGGTGTTGTGGCGGCGTTAAAGGTGTGTTGGGTGCTGCCCCGACC
CCAGCTGACCGCAAG**ACTGCATGCACCTTGCCTGAAATCAGCTGCTTATGCTATTAAGGGTATT**
AATATGGGCAAAGCCGCTGGACTTCCTAGTGCTTGTGGCGTTAACATTCCCTACAAGATCAGT
CCCTCTACTGACTGCTCCAGGGTCCAGTGA

Figure 11: Verified Sequence of SIP470 showing 141bp long sequence selected for homolog silencing (Underlined).

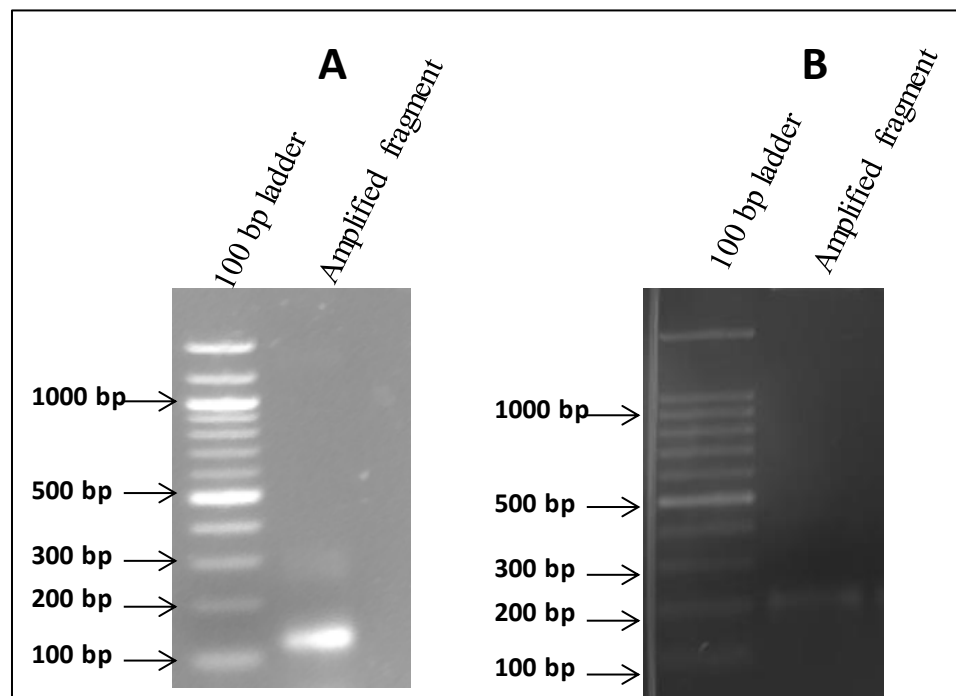


Figure 12: Agarose gel electrophoresis of the amplified silencing construct. **A.** PCR amplified fragment for the silencing fragment. **B.** PCR amplified fragment for homolog silencing. Asterisk shows the expected size of the band expected.

Purification of Resulting PCR Product

The amplified fragments were gel extracted and purified as described in the methods section. Results show the purified fragment for silencing (Figure 13A) and homolog silencing (Figure 13B) used for BP recombination reaction.

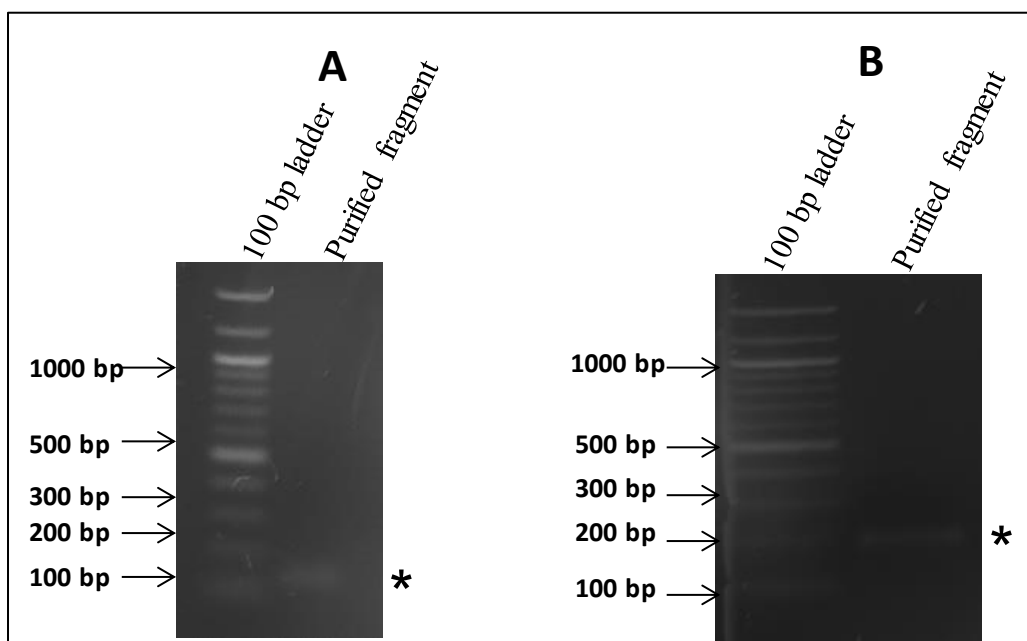


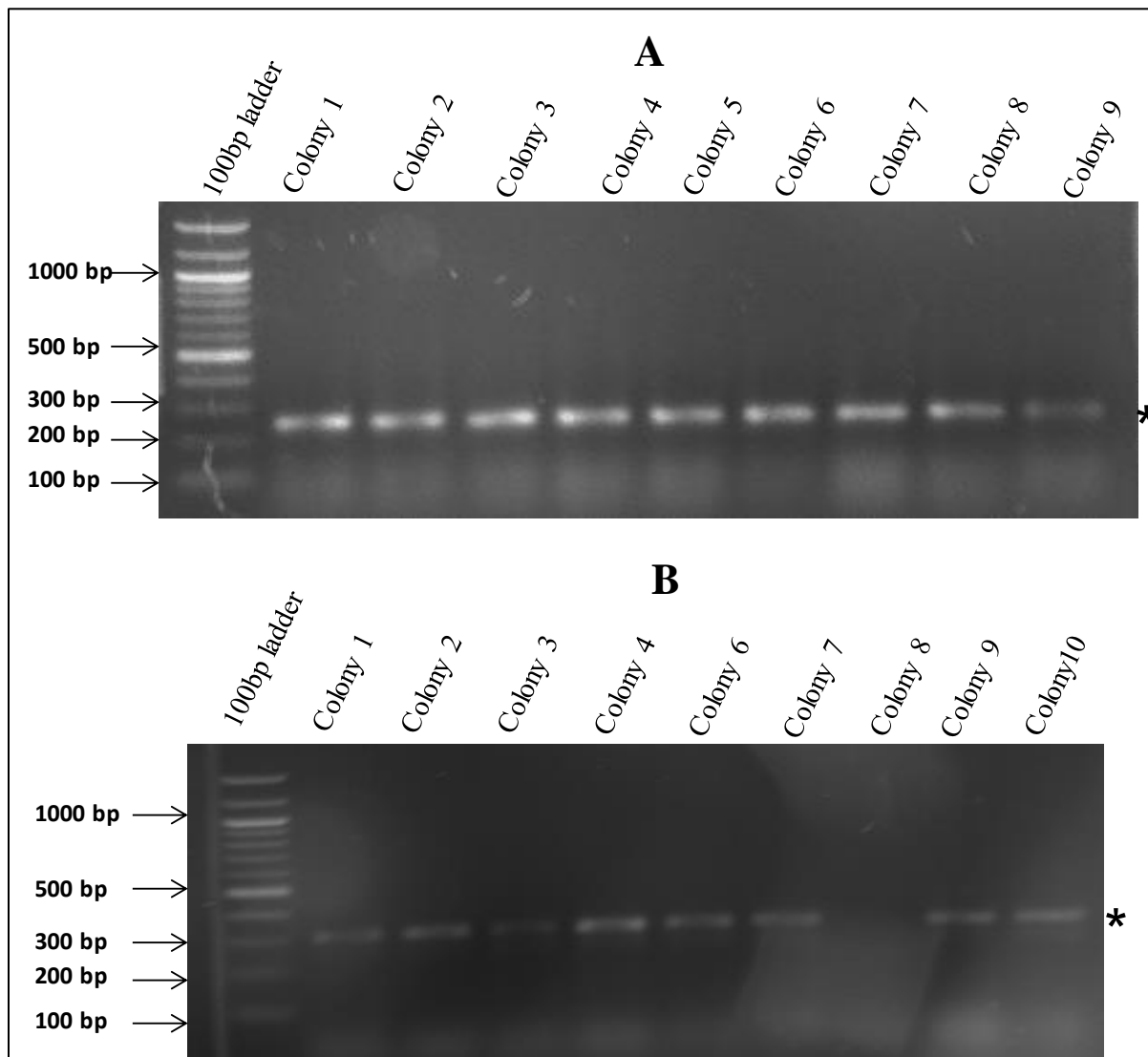
Figure 13: Agarose gel electrophoresis of the purified fragment. A. PCR purified fragment for silencing. B. PCR purified fragment for homolog silencing. Asterisk shows the expected size of the fragment expected.

BP Recombination Reaction

The DNA fragment obtained from gel extraction and purification was then used for the BP reaction. The BP reaction allows for the transfer of DNA fragments with attB1 and attB2 site into the entry clone (pDONR221) which contains attP1 and attP2 sites. The reaction was carried out as described in the methods section. After BP reaction, 1 μ l of the product was then used to transform competent *E. coli* cells as described in the methods.

Screening of Transformed Cells Using Colony PCR

Transformed colonies were selected and screened for the presence of *SIP470* silence fragment in the entry clone (pDNOR221) before further cloning steps. Results show the presence of *SIP470* fragment in the entry clone for silencing (Figure 14A) and homolog silencing (Figure 14B) respectively.



*Figure 14 : 1.5% EtBr stained agarose gel of colony PCR. A. Shows the presence of entry vector and silencing fragment for *SIP470*. B. shows the presence of entry vector and homolog silencing fragment for *SIP470**

Sequencing of Recombinant Plasmid

Plasmids from Five bacterial clones that were positive from colony PCR each from silencing and homolog silencing was isolated (as described in the methods section) and sent for sequencing. Results show a 100 percent match of the silencing fragment in the vector when aligned with a verified template (Figure 15).

SBIP470	-----TGTGGCGGCGTTAAAGGTGTGTGGGTGCTGCCCGGACCCAG	43
SILENCING1PDONOR221FORWARDM13	AGGCTATTGTGGCGGCGTTAAAGGTGTGTGGGTGCTGCCCGGACCCAG	50

SBIP470	CTGACCGCAAGACTGCA-----	60
SILENCING1PDONOR221FORWARDM13	CTGACCGCAAGACTGCAGGGAAGAGCTTCTCCGCTGCCTAGAATCTGCA	100

SBIP470	--	
SILENCING1PDONOR221FORWARDM13	TT	102

Figure 15: Sequence Alignment of the silencing fragment in entry clone pDONR221 with the selected 60bp sequence for silencing. Perfectly matching sequences are indicated with a star below the sequence. The M13 forward primer was used for DNA sequencing.

LR Recombination Reaction

The verified sequence in the entry clone was then ligated into the destination vector pHELLSGATE 8. The LR reaction allows the transfer of DNA sequences of interest flanked by attL1 and attL2 in the entry vector into the destination vector pHELLSGATE 8 with attR1 and attR2. The reaction was carried out as described in the methods section. After LR reaction, 1 μ l of the product was then used to transform competent *E.coli* cells as described in the methods.

Screening of Transformed Cells Using Colony PCR

Transformed colonies were selected and screened for the presence of *SIP470* silencing fragment in the destination vector (pHELLSGATE 8). Results show the presence of *SIP470* fragment in the destination clone for silencing (Figure 16). The same procedure as followed for homolog silencing but gel pictures were not taken because the results from silencing experiments validated the process.

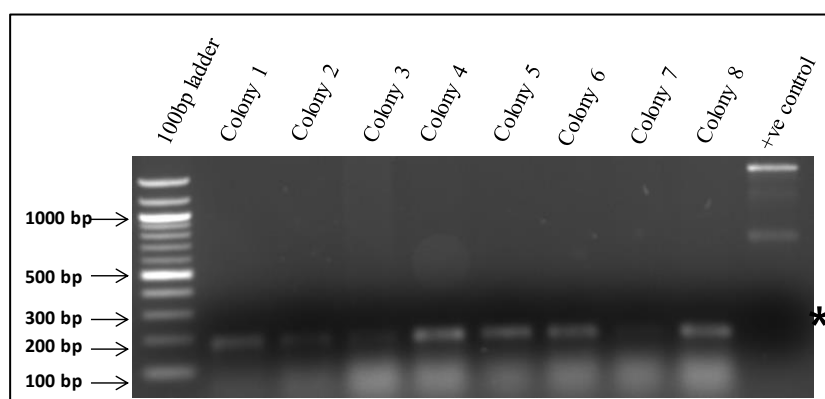


Figure 16: 1.5% EtBr stain agarose gel of colony PCR. Figure showing the presence of destination vector and silencing fragment for SIP470.

Recombinant pHELLSGATE 8-*SIP470* Plasmid DNA Isolation

Colony # 4 (Figure 16) was subcultured in LB media with the appropriate antibiotics overnight and the plasmid was extracted (as described in the methods section). To further verify the presence of silencing fragment in the destination vector (pHELLSGATE 8), a PCR amplification was done using the extracted plasmid as a template and result shows the presence of the silencing fragment of interest (Figure 17). One μ l of plasmid DNA for silencing (247ng/ml) and homolog silencing (150 ng/ml) was later used to transform electrocompetent LBA 4404 cells respectively.

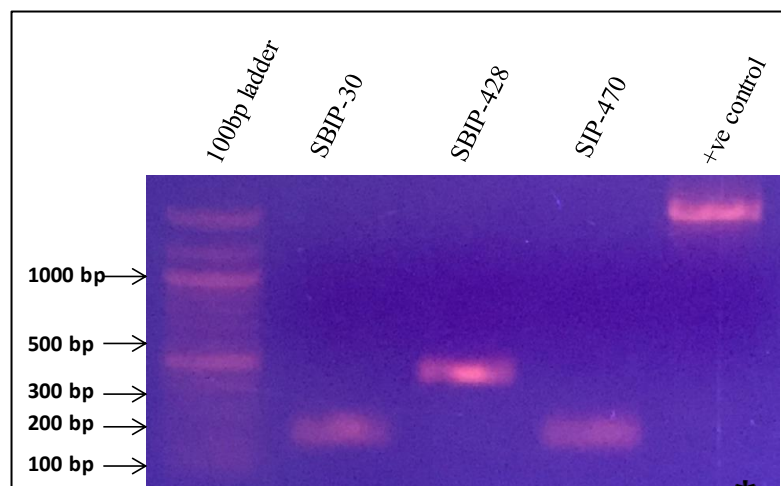


Figure 17: 1.5% EtBr stained Agarose Gel. Figure showing the PCR silencing fragment for SIP470. DNA fragment of interest.

Transformation of pHELLSGATE8-SIP470 into LBA 4404

Transformed LBA4404 was screened for the presence of pHELLSGATE8-SIP470 using colony PCR as earlier described. The result shows the presence of pHELLSGATE8-SIP470 in Agrobacterium (Figure 18).

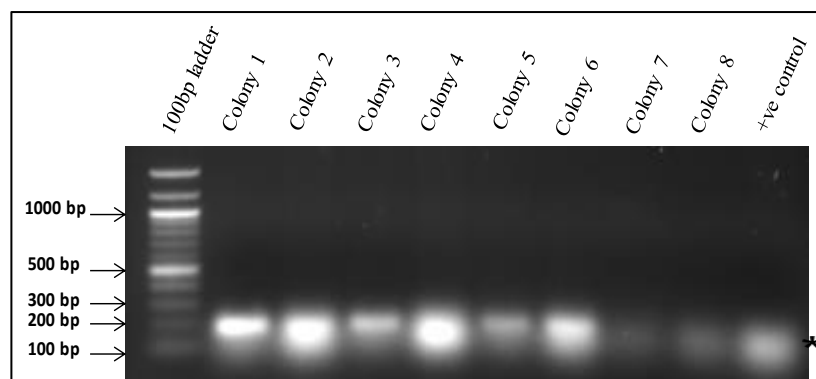


Figure 18: Agarose gel showing the presence of pHELLSGATE8-SIP470 in Agrobacterium.

Generating SIP470 Overexpression Transgenic Tobacco Lines

In order to characterize the functional role of SIP470 in plants in biotic and abiotic stresses, there was a need to generate a transgenic line that overexpresses SIP470 protein of interest in order to study the possibility of a reversed and even enhanced role in the silenced plants (complementation). Overexpressed lines of SIP470 were generated using an inducible system as explained in the material and methods section. We used pGEMT as the entry vector and pER8 (Figure 19) as the destination vector. Verified clone in the destination vector was transformed into competent *Agrobacterium tumefaciens* LBA4404 cells which were used for the plant transformation.

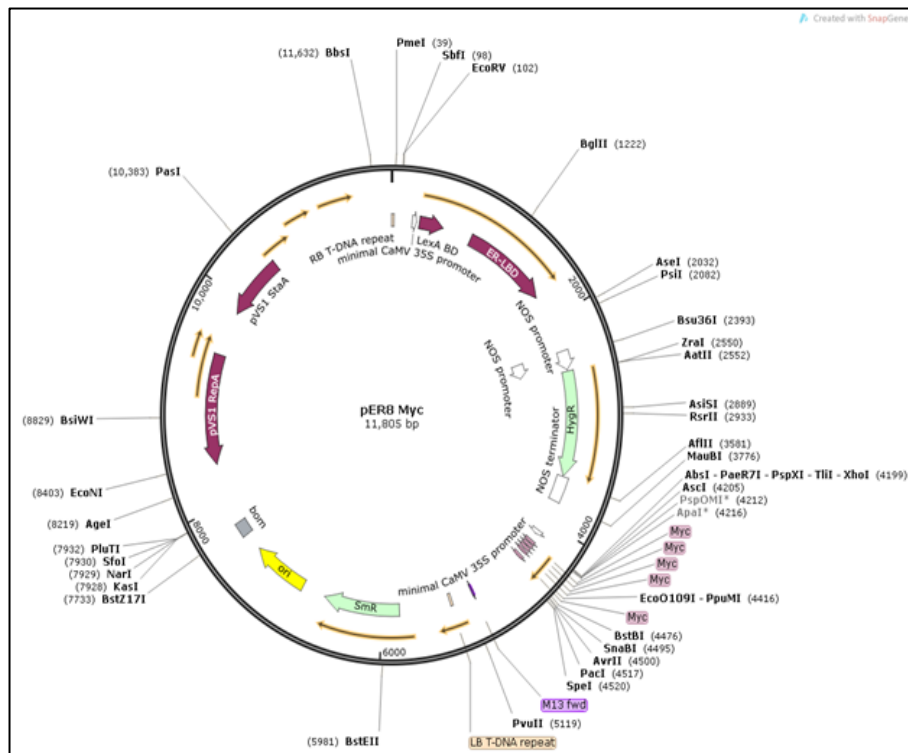


Figure 19: Vector Map of *pER8* used in generating overexpressed SIP470 transgenic lines.

PCR amplification of *SIP470* Coding Sequence

To generate the overexpression transgenic lines, *SIP470* coding sequences (Figure 20) was amplified from a verified template (pDONR221+470 clone number P2, box 4) using a primer designed to incorporate an Avr II and Spe I enzyme site at the C and N terminal of *SIP470* gene respectively. PCR amplification was then performed as earlier described in the methods section. Results show a successful amplification of *SIP470* coding sequence (Figure 21).

```
ATGGAAATGGTTGGCAAGATTGCGTGCTTTGTGTTACTTTGCATGGTGGTGGTTGCA
CCCCATGCAGAGGCACTGAGCTGCGGCCAGGTTCACTCTGGCCTGGCTCTTTGCCCTC
CCTTATCTGCAGAGTCGCGGCCCTCTAGGGAGGTGTTTGTGGCGGCGTTAAAGGTGTG
TTGGGTGCTGCCCCGACCCCAGCTGACCGCAAGACTGCATGCACTTGCCTGAAATCA
GCTGCTTATGCTATTAAGGGTATTAATATGGGCAAAGCCGCTGGACTTCCTAGTGCT
TGTGGCGTTAACATTCCCTACAAGATCAGTCCCTCTACTGACTGCTCCAGGGTCCAG
TGA
```

Figure 20: Verified Sequence of SIP470 showing 345bp long coding sequence selected for overexpression (underlined).

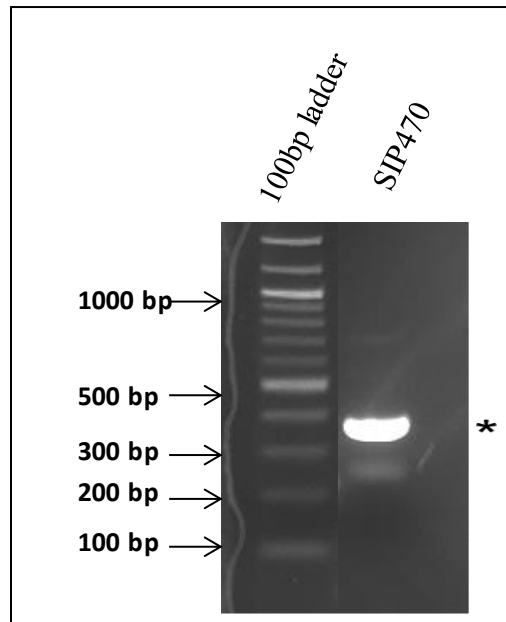


Figure 21: Agarose gel showing the PCR amplified fragment for SIP470 overexpression.

Screening of Transformed Cells in pGEMT Using Colony PCR

The amplified fragment was purified as described in the methods section. The purified fragment was then ligated into pGEMT entry vector as described in the materials section. The ligation mixture was then used to transform competent *E.coli* Top10 cells. Transformed colonies were selected and screened for the presence of *SIP470* coding sequence and the result shows the presence of *SIP470* coding sequence in the pGEMT vector (Figure 22).

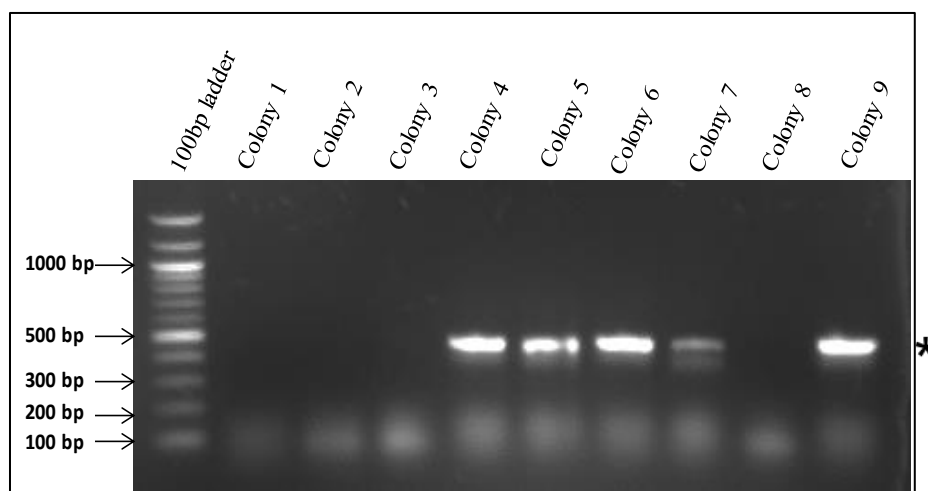


Figure 22: 1.5% EtBr stain agarose gel. Figure showing the presence *SIP470* in pGEMT

Sequencing of Recombinant pGEMT-*SIP470* Plasmid

Plasmids from three bacterial clones that were positive from colony PCR each for overexpression was isolated (as described in the methods section) and sent for sequencing. Results show a 100 % match of the silencing fragment in the vector when aligned with a verified template (Figure 23).

SBIP470	-----ATGGAAATGGTTGGCAAGATTGCGTGCTTTGTGTT	35
TM1-pGEMT	GGCCGCGGGAATTCGATTCCTAGGTATGGAAATGGTTGGCAAGATTGCGTGCTTTGTGTT	60

SBIP470	ACTTTGCATGGTGGTGGTTGCACCCCATGCAGAGGCACTGAGCTGCGGCCAGGTTCAGTC	95
TM1-pGEMT	ACTTTGCATGGTGGTGGTTGCACCCCATGCAGAGGCACTGAGCTGCGGCCAGGTTCAGTC	120

SBIP470	TGGCCTGGCTCTTTGCCTCCCTTATCTGCAGAGTCGCGGCCCTCTAGGGAGGTGTTGTGG	155
TM1-pGEMT	TGGCCTGGCTCTTTGCCTCCCTTATCTGCAGAGTCGCGGCCCTCTAGGGAGGTGTTGTGG	180

SBIP470	CGGCGTTAAAGGTGTGTTGGGTGCTGCCCGGACCCAGCTGACCGCAAGACTGCATGCAC	215
TM1-pGEMT	CGGCGTTAAAGGTGTGTTGGGTGCTGCCCGGACCCAGCTGACCGCAAGACTGCATGCAC	240

SBIP470	TTGCCTGAAATCAGCTGCTTATGCTATTAAGGGTATTAATATGGGCAAGCCGCTGGACT	275
TM1-pGEMT	TTGCCTGAAATCAGCTGCTTATGCTATTAAGGGTATTAATATGGGCAAGCCGCTGGACT	300

SBIP470	TCCTAGTGCTTGTGGCGTTAACATTCCTACAAGATCAGTCCCTCTACTGACTGCTCCAG	335
TM1-pGEMT	TCCTAGTGCTTGTGGCGTTAACATTCCTACAAGATCAGTCCCTCTACTGACTGCTCCAG	360

SBIP470	GGTCCAGTGA-----	345
TM1-pGEMT	GGTCCAGTGAACCTAGTAATCACTAGTGAATTC	392

Figure 23: Sequence Alignment of SIP470 overexpression clone in pGEMT (TM1-pGEMT). *Perfectly matching nucleotides are indicated with a star below the sequence.*

Ligation of Digested pGEMT-470-4 and pER8

Sequence verified clone (pGEMT-SIP470-4) and pER8 plasmid were then restricted digested as described in the methods section. The ligated mixture was then transformed into *E. coli* and incubated overnight. Plasmid from transformed bacteria was isolated and analyzed on a 1.5% EtBr agarose gel. The result shows successful ligation of SIP470-pER8 (Figure 24).

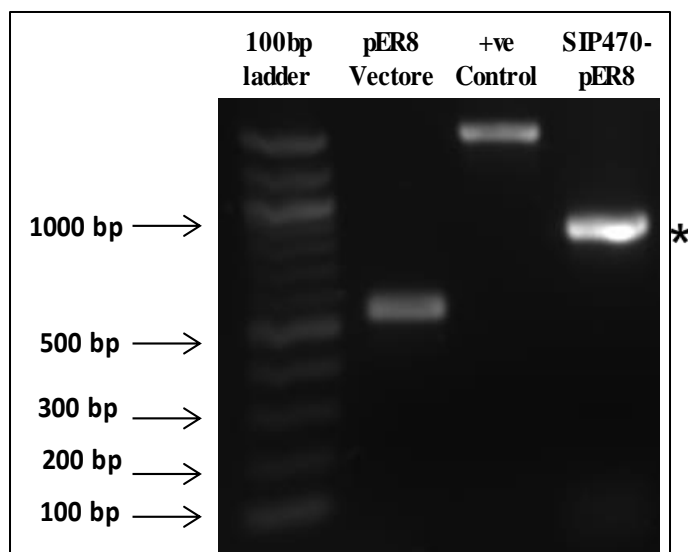


Figure 24: 1.5% EtBr stain agarose gel. Figure showing the presence of ligated SIP470-pER8 having a higher size approximately 800bp than the pER8 empty vector.

Sequencing of Recombinant *SIP470*-pER8 Plasmid

SIP470-pER8 clone from the above was then sent for sequencing. Analysis of the sequence obtained shows that the translated protein of *SIP470* is in the right frame of the pER8 vector as seen in Figure 25.

```

atggaqcaaaaagctcatttctgaaagaggaacttqaatgaaatggaq
M E Q K L I S E E D L N E M E
caaaaagctcatttctgaaagaggaacttqaatgaaatggaqcaaaaag
Q K L I S E E D L N E M E Q K
ctcatttctgaaagaggaacttqaatgaaatggaqcaaaaagctcatt
L I S E E D L N E M E Q K L I
tctgaagaggacttgaatgaaatggagagcttggggcgacctcacc
S E E D L N E M E S L G D L T
atggagcaaaaagctcatttctgaaagaggacttgaattcggtaccc
M E Q K L I S E E D L N S V P
cqqgttcgaaatcgaatggaatcctacgaacgaatggaatggaatgga
R V R N R W I L R S L G M E M
gttggaagattgagctgctttgtgttactttgcatggtggtggtt
V G K I A C F V L L C M V V V
gcaccccatgcagaggaactgagctgcggccaggttcagtctggc
A P H A E A L S C G Q V Q S G
ctggctccttgcctcccttatctgcagagtcgcggccctctaggg
L A L C L P Y L Q S R G P L G
aggtgttggtggcggttaaaggtgtgttggtgtgtgcccggacc
R C C G G V K G V L G A A R T
ccagctgaccgcaagactgcagtcacttgccctgaaatcagctgct
P A D R K T A C T C L K S A A
tatgctattaaggtattaatatggaagccgctggaacttcct
Y A I K G I N M G K A A G L P
agtgtgtggtggttaacattccctacaagatcagtcctctact
S A C G V N I P Y K I S P S T
gactgctccaggttccagtga 735
D C S R V Q *

```

Figure 25: Figure shows a translated protein sequence of SIP470 (in red) in the frame as indicated by the translated sequence upstream (in black).

Transient Expression of SIP470-pER8 in *Nicotiana benthamiana*

The verified SIP470-pER8 clone was transformed into competent *Agrobacterium* cells as described in the methods section. In order to verify whether SIP470-pER8 clone was able to express SIP470 protein when induced, transient expression was carried out as described in the methods section. Figure 26 shows a western blot detection of SIP470 transiently expressed in the leaves of the tobacco plant. This process was important to determine the successful expression of the protein of interest after plant transformation.

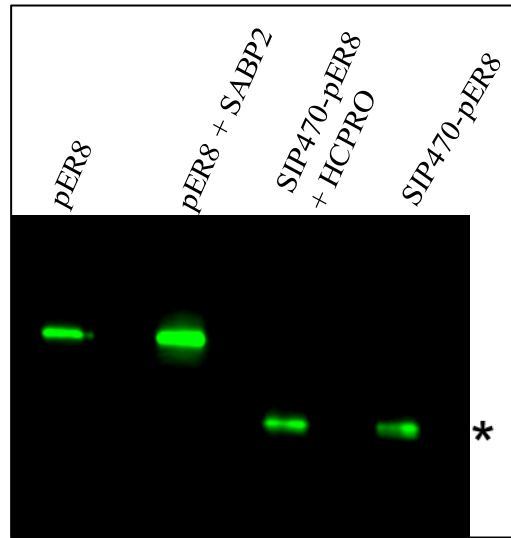


Figure 26: Western blot analysis. This shows SIP470 protein from transient expression in *Nicotiana benthamiana*. Protein was detected by using myc tag antibody.

Screening of Transgenic Line for Silencing and Homolog Silencing

Plant transformation was carried out as described in the materials and methods section. Leaf tissues were collected from transformed plants to check the relative expression of SIP470 in the transgenic silenced and homolog silenced lines respectively when compared to the wild type plant. mRNA was extracted from the leaf tissues collected from silenced as well as homolog silenced lines as described in the methods section. The corresponding cDNA was synthesized from the resulting mRNA and an RT-PCR was done using the screening primer. Results from RT-PCR shows a successfully silenced SIP470 (Figure 27A) as well as its homologs (Figure 27B) as seen by the inability to detect the screening sequence in the transgenic lines.

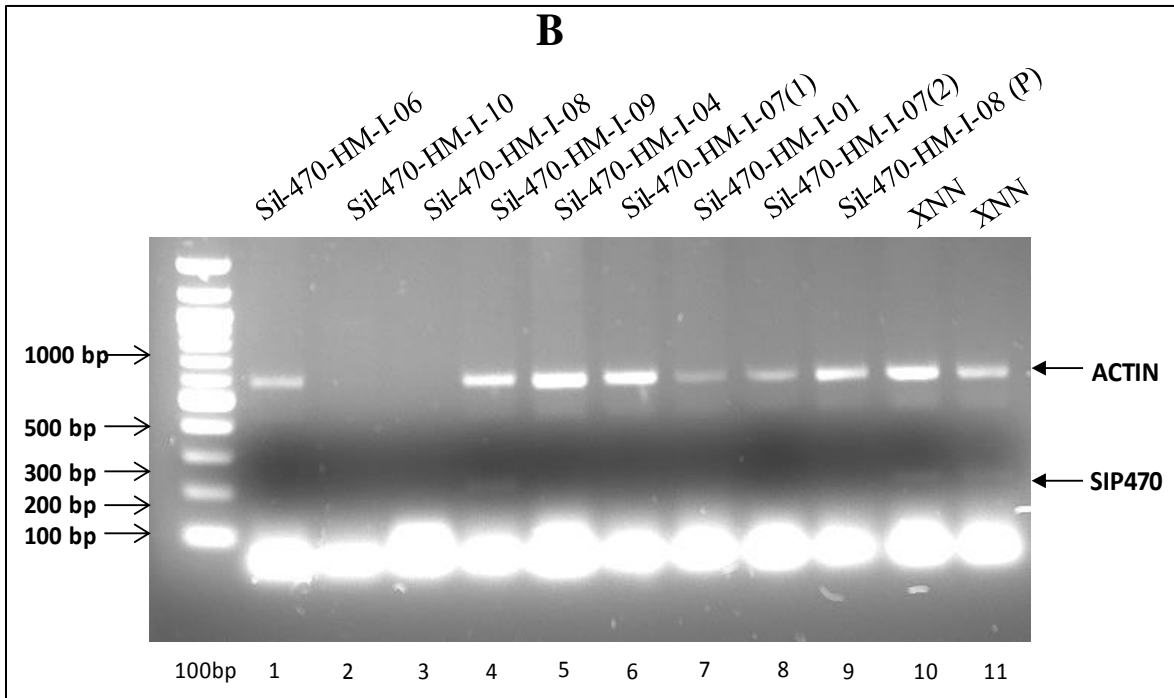
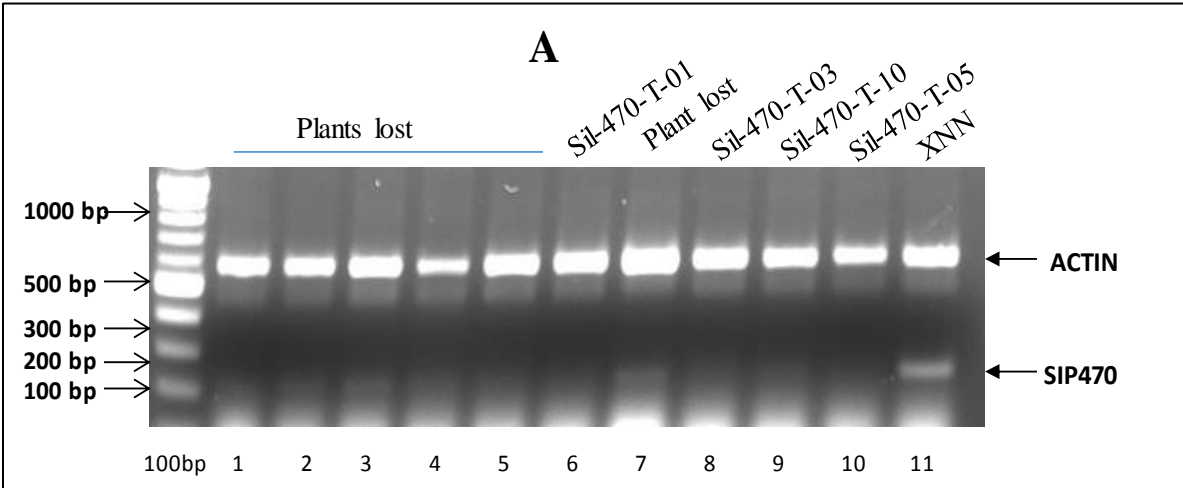


Figure 27: 1.5% EtBr stain agarose gel. A. shows RT-PCR results for silenced plants and their relative level of expression of SIP470 with XNN having a relatively higher expression level when compared to the silenced lines. B. shows RT-PCR results for homolog silenced plants and their relative level of expression of SIP470

Plant transformation was done as earlier described. Successfully transformed plants for overexpression were subjected to screening by inducing leaf disc samples to express SIP470 using β estradiol solution. Overexpression of SIP470 was not detected using this method of screening as shown by the inability to detect SIP470 protein from the western blot analysis using C-myc tag (Figure 28A and Figure 28B)

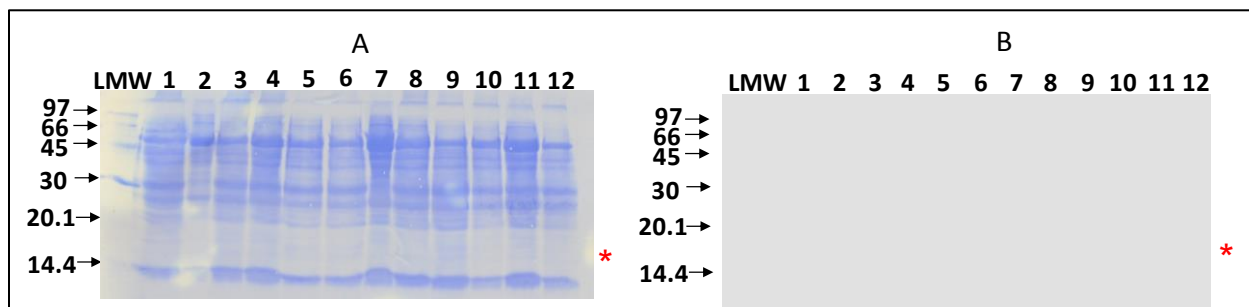
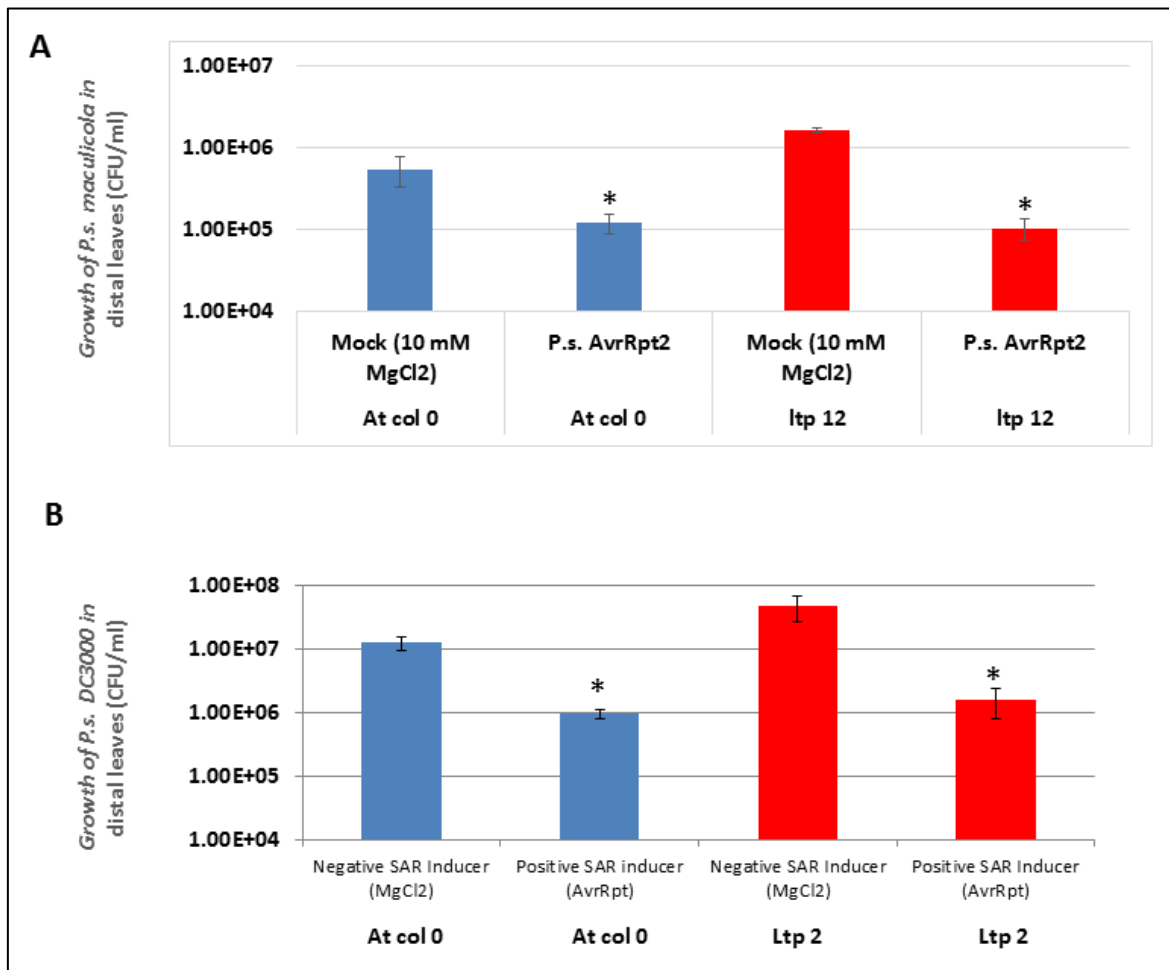


Figure 28: SDS-PAGE and western blot analysis. A. Coomassie stained blot showing various leaf disc samples screened for SIP470 overexpression B. Western blot analysis various leaf disc samples screened for SIP470 overexpression.

Homologs of SIP470 in *Arabidopsis* are not Defective in Systemic Acquired Resistance (SAR)

In order to investigate the role of SIP470 in inducing SAR in plants, Tobacco SIP470 translated protein sequence was used to search for similar proteins in *Arabidopsis* species. *Arabidopsis* ltp2 has the highest similarity with SIP470 with a 53% match while *ltp12* has a 46% similarity with SIP470 (Chapagai, 2014). Knockout mutants for *ltp12* and *ltp2* were obtained from the Biological Resource Center, OH, USA, screened (Chapagai, 2014) and was used for investigating the possible role of *ltp12* and *ltp2* in inducing SAR. SAR experiments were carried out as described in the materials and methods section. Results show that both *ltp12* (Figure 29A) and *ltp2* (Figure 29B) were capable of inducing acquired resistance to pathogens.



*Figure 29: SAR is not affected in ltp2 and ltp12 mutants of Arabidopsis thaliana plants. A. SAR was not affected in ltp12 mutant Arabidopsis thaliana plants. B. SAR was not affected in ltp2 mutant Arabidopsis thaliana plants. A significant difference was observed between mock and avirulent treated samples (t-test, $P < 0.05$). 2 lower leaves of mutant Arabidopsis plants were first treated with avirulent pathogen (OD=0.002) (P.S tomato DC3000 AvrRPT2). After 2 days post infection (2 DPI), three upper leaves were challenged with a virulent pathogen (OD=0.0002) *Pseudomonas syringe* pv. *maculicola*. Control plants were infiltrated using 10mM MgCl₂.*

Homologs of SIP470 in *Arabidopsis* are Defective in Basal Resistance.

The role of SIP470 homologs was also investigated for the basal resistance to pathogens in comparison to the wild type plant col-0 as described in the materials and methods section. Results show that *ltp12* mutant plants are significantly more susceptible to bacteria pathogen (Figure 30A and 30B) allowing more growth of bacteria at 3 dpi while *ltp2* didn't show a significant difference in susceptibility to the pathogen (Figure 29C).

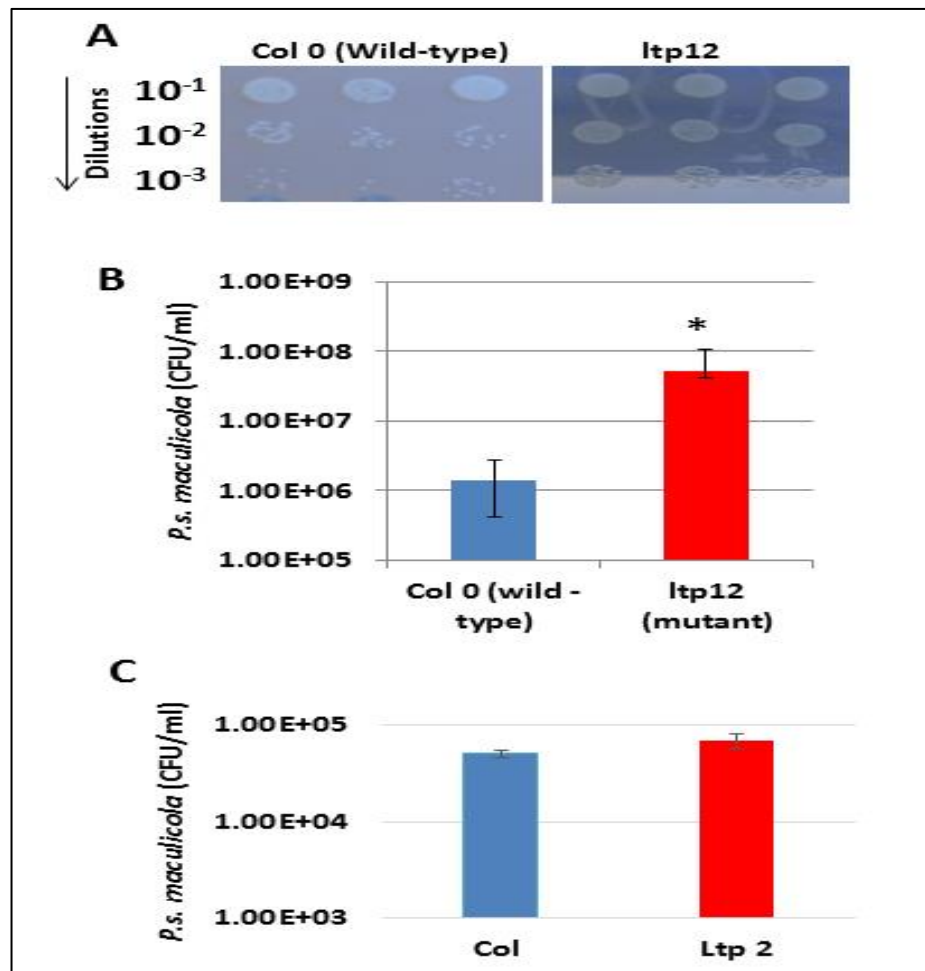


Figure 30: Basal Resistance in *Arabidopsis* wild-type *col 0*, *ltp12* and *ltp2*. **A.** shows the serial dilution of leaf extract of *P.s. maculicola* infected plants at 3 days post infection for *ltp12* and *Col 0*. **B** Growth of *P.s. maculicola* in *ltp12* and *Col 0* at 3 days post infection. A significant difference was observed between the growth of bacteria in *ltp12* and *Col 0* (t-test, $P < 0.05$). **C.** The growth of *Ps maculicola* in *ltp2* and *Col 0* at 3 days post infection. No significant difference was observed.

Role of SIP470 in Abiotic Stress

Ltp12 mutant in *Arabidopsis* is Susceptible to Salt Stress (NaCl)

To determine if SIP470 could play a role in abiotic stress, a ltp12 mutant that was defective in basal resistance was grown in the presence of varying concentration of NaCl as described in the materials and method section. Results show that ltp12 mutants are more susceptible to salt stress at 150mM concentration when compared to the control (Col 0) as seen by the bleaching leaves (Figure 31C). No leave bleaching effect was observed at 50mM NaCl concentration (Figure 31B) but the plants appeared to be growth retarded when comparing the growth with the control plates (Figure 31A) while the 200mM concentration affected both ltp12 mutant and Col 0 with both plant type showing a lot of leave bleaching but ltp12 being more affected by salt stress (Figure 31C).

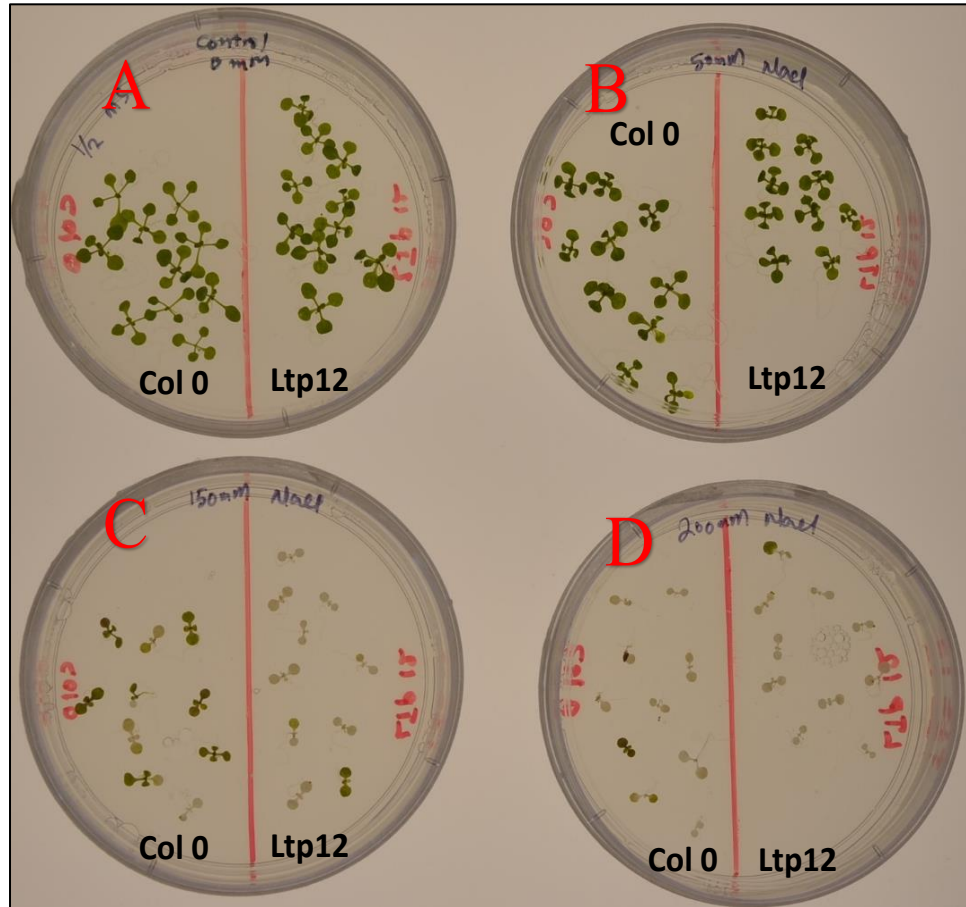


Figure 31: Effect of NaCl on 15 days old seedlings from *ltp12* mutant and Col 0. 10-day old seedlings of Arabidopsis *ltp12* mutant and Col 0 were transferred into a half strength MS plate containing A. no NaCl incorporated in half MS. B. Plates containing 50mM NaCl. C. plates containing 150mM NaCl. D. plates containing 200mM NaCl.

Ltp12 Mutant in Arabidopsis Susceptible to Oxidative Stress (H₂O₂)

The effect of oxidative stress on the *ltp12* mutant plant was also investigated using hydrogen peroxide H₂O₂ incorporated in the MS media. The experiment was carried out as described in the materials and methods section. Results show that *ltp12* mutant was affected by 3mM hydrogen peroxide as seen by the bleaching in the leafs when compared to the control (Figure 32).

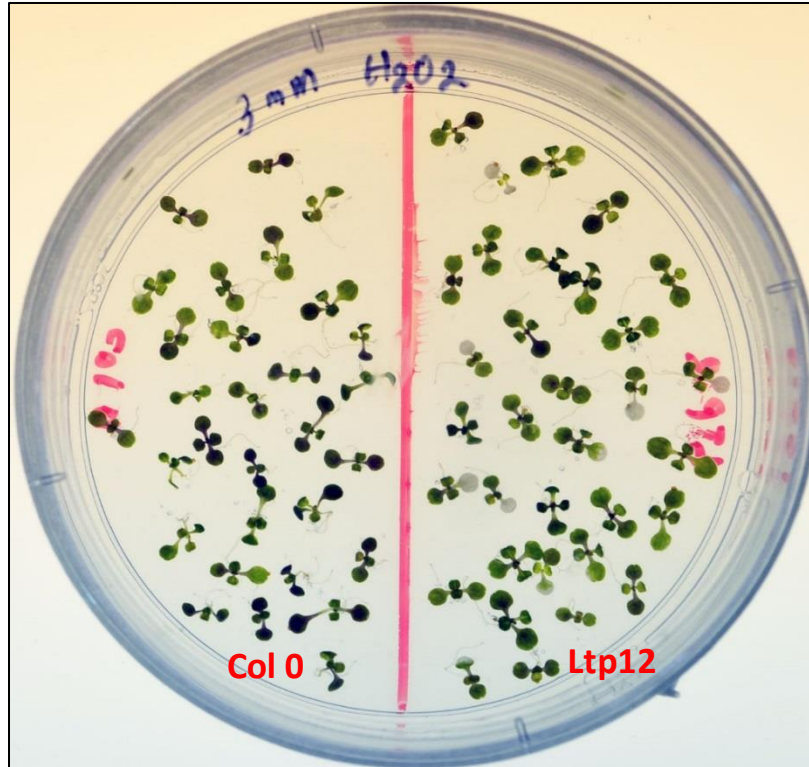


Figure 32: Effect of 3mM H₂O₂ on 15 days old seedling of ltp12 mutant and Col 0. 10-day old seedlings of Arabidopsis ltp12 mutant and Col 0 were transferred into a half strength MS plate containing 3mM hydrogen peroxide.

Ltp12 Mutant in *Arabidopsis* is Susceptible to Absciscic Acid (ABA)

The effect of ABA incorporated in the growth media was also investigated. The experiment was carried out as describe in the methods section. Results show that *ltp12* mutants are hypersensitive to 20 μ M ABA as seen by the bleaching of its leaves when compared to Col 0 with no visible bleaching on the leaves (Figure 33).

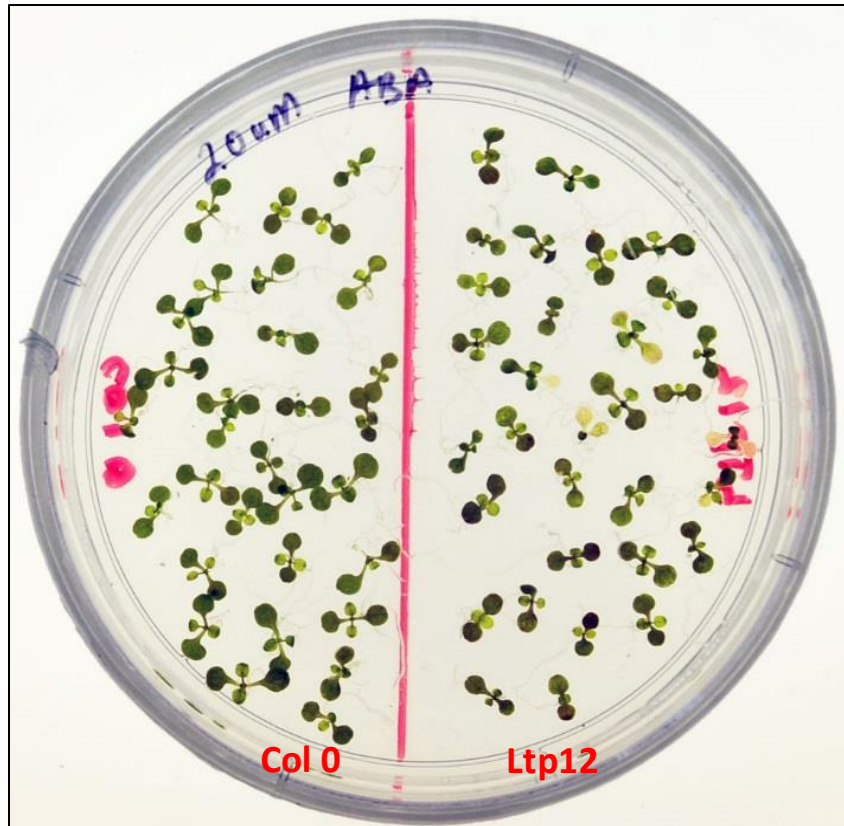


Figure 33: Effect of 20 μ M ABA on 15 days old seedling of *ltp12* mutant and Col 0. 10-day old seedlings of Arabidopsis *ltp12* mutant and Col 0 were transferred into a half strength MS plate containing 3mM hydrogen peroxide.

Ltp12 Mutant in *Arabidopsis* is Susceptible to Drought

The effect of drought on the survival of SIP470 homologs (*ltp12* and *ltp2*) were also investigated as described in the methods section. Figure 34C shows that *ltp12* mutants are susceptible to drought stress when compared to wild type Col 0 as *ltp12* mutant plants were not able to survive drought even after subsequent exposure to water. Col 0 and *ltp2* mutants were able to survive drought stress.

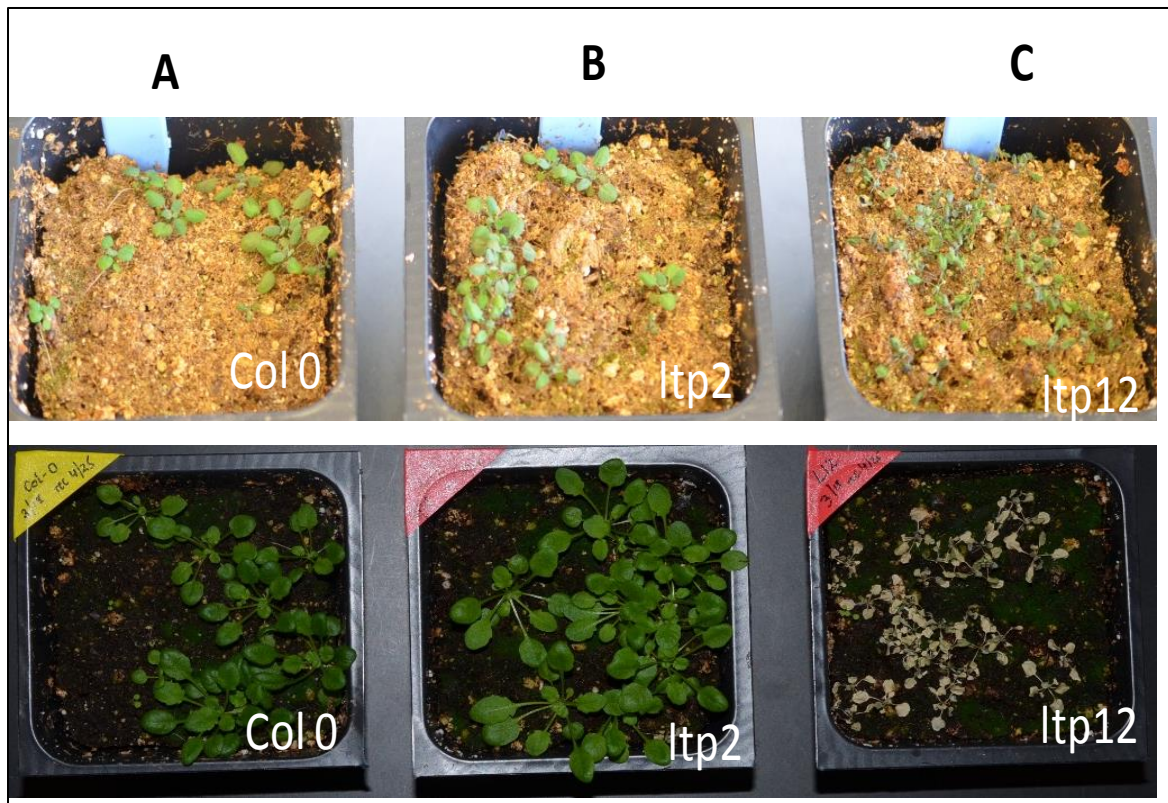


Figure 34: Effect of drought on seedlings of ltp12 mutant and Col 0. Seedlings of Arabidopsis ltp12 mutants were subjected to drought and then later watered.

Expression of PR1 Defense Gene on Exposure to Virulent pathogen

In order to understand at the molecular level what potential role ltp12 plays in basal defense signaling, gene expression studies of a defense gene (PR1) was carried out as outlined in the materials and methods section to determine if pathogenesis-related protein 1 (PR1) is up-regulated or down-regulated in ltp12 mutant Col 0 plants. Figure 35 shows the expression level of PR1 was delayed in ltp12 mutant plants but not in wild type (col 0) Plants on exposure to the virulent pathogen.

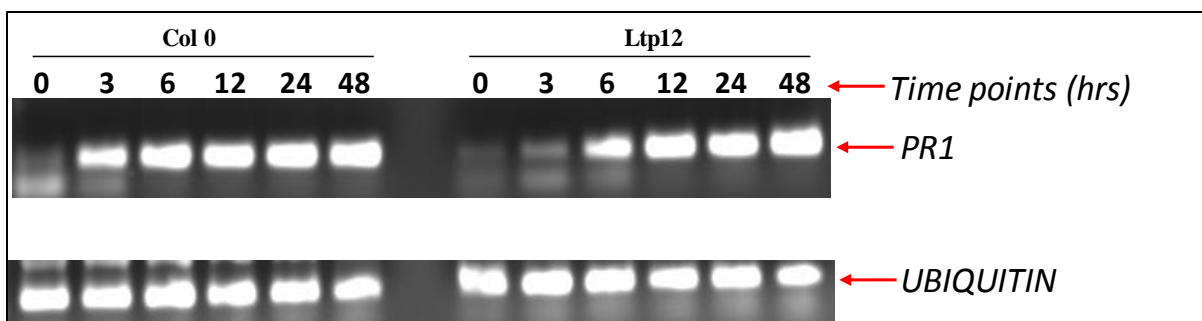


Figure 35: PR1 Gene Expression upon P.s. maculicola exposure. Expression of *PR1* on exposure to *P.s maculicola*. 0, 3, 6, 12, 24 and 48 represents the different time points of sample collection. cDNA was made from total RNA and used as a template to amplify PR1 and ubiquitin (internal control).

Tobacco SIP470 is a Lipid Transfer Protein (Hypothesis 2)

Expression of Recombinant SIP470 in *E. coli*

In order to determine if SIP470 is a lipid transfer protein as predicted from bioinformatics analysis, recombinant SIP470 was expressed in *E. coli* as described in the materials and methods section. Expression of recombinant SIP470 was detected by SDS-PAGE and western blot analysis using monoclonal Anti-polyhistidine antibody. There was a higher expression of recombinant SIP470 in pDest17-470 transformed in pLysE cells (Figure 36A and 36B). This clone was chosen for further experiments.

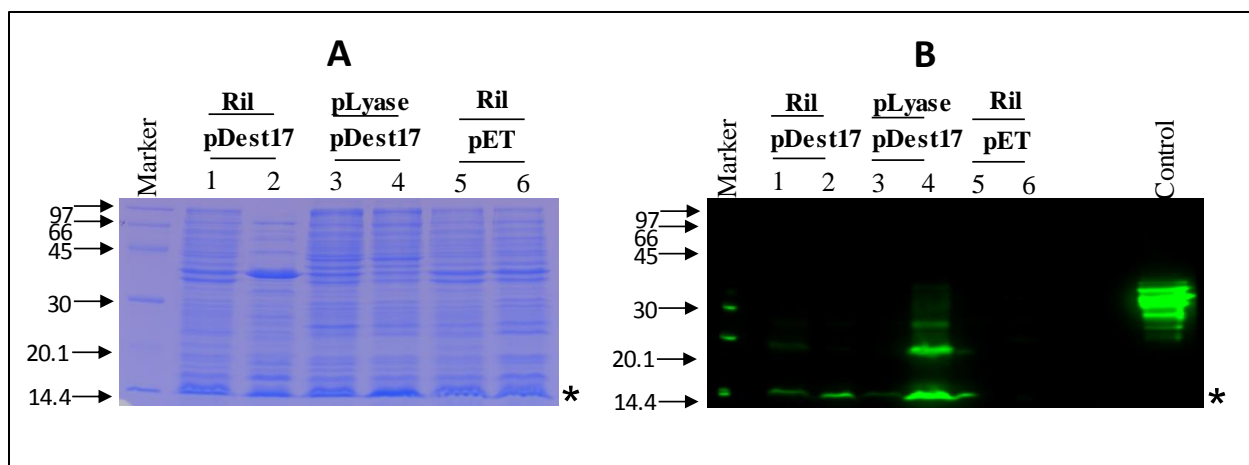


Figure 36: Expression of recombinant *SIP4670*. **A.** SDS page analysis of different clones of *SIP470* in different protein expression cell lines. **B** Western blot analysis of recombinant *SIP470* detected using poly-Histidine antibody. Lanes 1, 3 and 5 are un-induced, lanes 2, 4 and 6 are induced using 10 μ M IPTG. Asterisk represent the expected position of *SIP470* protein on the blot

Solubility Test

In order to get soluble fractions of recombinant *SIP470* for biochemical assay, a solubility test was done as described in the methods section. Resulting pellet collected from a 1ml overnight culture grown at different temperatures (17°C and 37°C) were re-suspended in 1X Ni-NTA binding buffer and the cells were broken by centrifugation. Both the soluble (supernatant) and insoluble (pellet) fractions were collected and analyzed using SDS page and western blot. From the result, it was clear that recombinant *SIP470* was more expressed at 37°C in its insoluble and soluble form compared to 17°C (Figure 37).

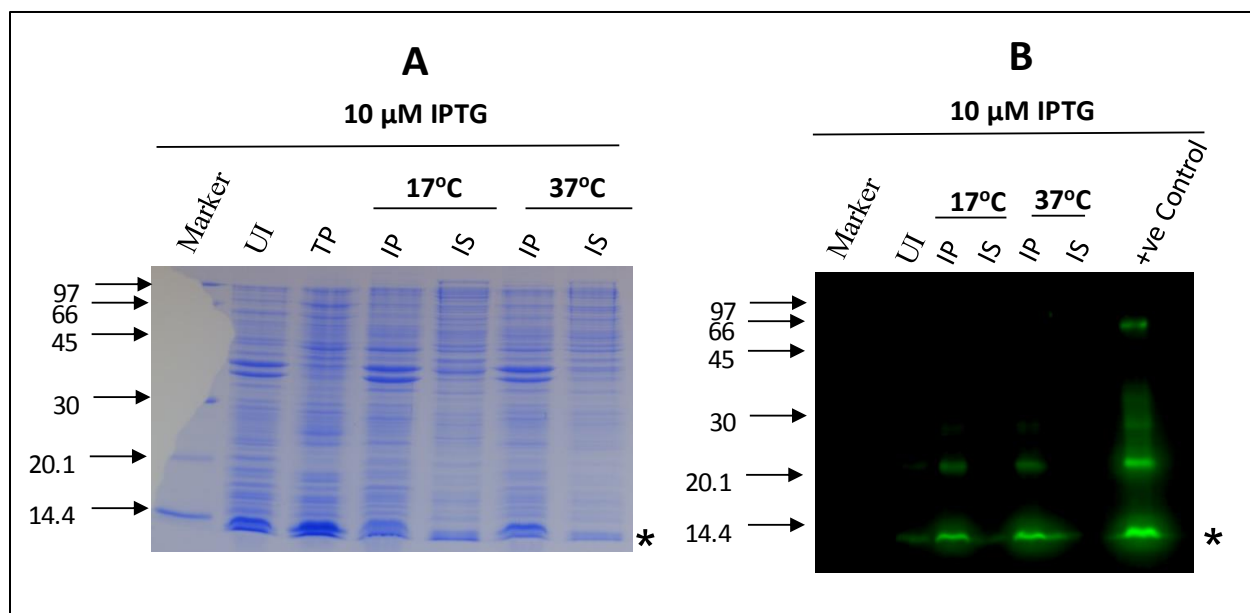


Figure 37: Expression of recombinant SIP470. A. SDS page analysis of SIP470 in pLysE cell line at a different temperature of expression. *B.* Western blot analysis of recombinant SIP470 detected using poly-Histidine antibody. ‘UI’ in this figure stands for un-induced, ‘TP’ for total protein, ‘IP’ for induced pellet, IS for induced supernatant and the asterisk represent the expected position of SIP470 protein on the blot

Purification of Recombinant *SIP470* using Ni-NTA Column Affinity Chromatography

Expressed recombinant SIP470 was purified using Ni-NTA affinity chromatography as described in the materials and methods section. Samples were eluted with 250mM imidazole. Eluted fractions were mixed with 6X SDS loading dye, boiled for 6 minutes and centrifuged. Resulting supernatant from the centrifugation was analyzed using SDS page and western blot. Result shows the successful detection of recombinant SIP470 using western blot (Figure 38)

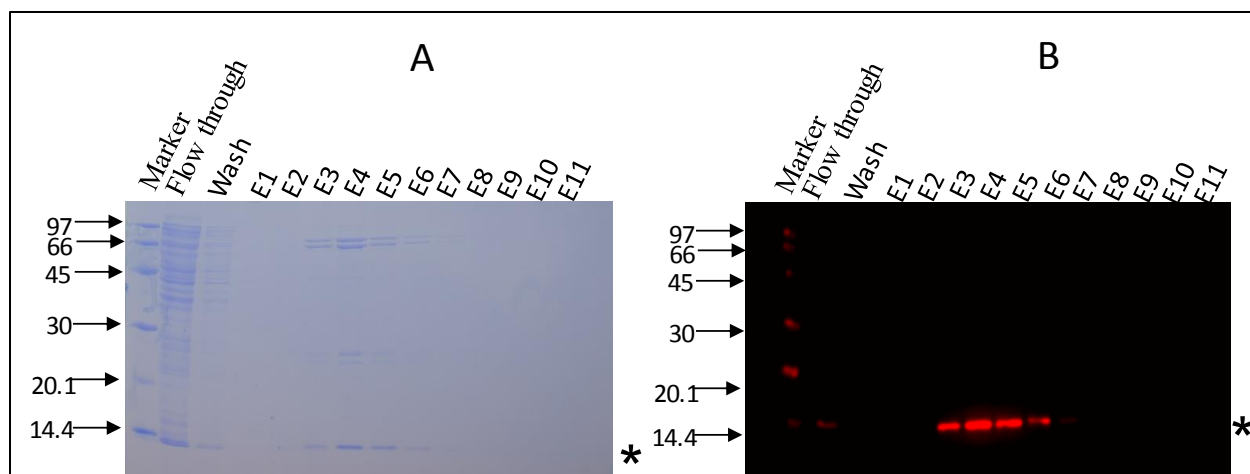


Figure 38: Purification of recombinant SIP470 using NI-NTA affinity chromatography. A. SDS page analysis of purified recombinant SIP470 with eluted fractions E1-E11. **B** Western blot analysis of recombinant SIP470 detected using poly-Histidine antibody. The asterisk represents the expected position of SIP470 on the blot, E1-E11 represents each eluted fractions.

Biochemical Characterization

Lipid Binding Assay

In order to test if SIP470 is a lipid transfer protein, an *in vitro* lipid binding assay was performed using soluble Ni-NTA purified SIP470 and a fluorescent lipid binding substrate called TNS (*p*-toluidinonaphthalene-6-sulfonate) as described in the material and method section. Under experimental conditions, recombinant SIP470 binds to TNS lipophilic probe as indicated by the increasing fluorescence with an increasing concentration of TNS (Figure 39). Fluorescence data was measured by subtracting from the control.

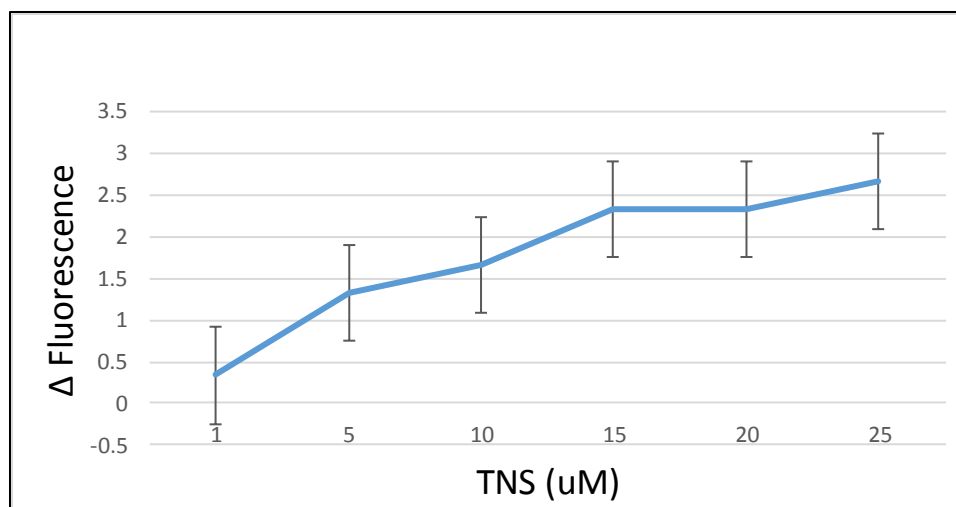


Figure 39: Recombinant purified SIP470 binds the Lipophilic Probe TNS. Recombinant SIP470 was expressed without the signal peptide and then purified in *E. coli* using NI-NTA affinity chromatography. Purified SIP470 incubated with increasing concentration of TNS shows an increasing fluorescence activity

Effect of SABP2 on Binding Affinity

The effect of SABP2 on lipid binding activity of SIP470 was also carried out as described in the materials and methods section. A 100μl reaction which was composed of a fixed concentration of SIP470 and varying concentrations of SABP2 was made. Results show a reduction in the binding affinity of SIP470 in the presence of SABP2 (Figure 39)

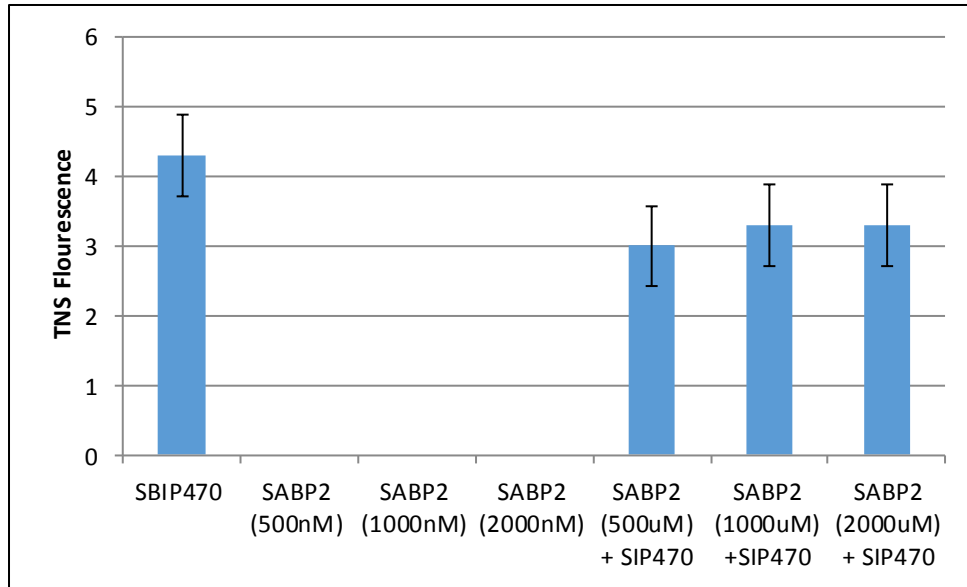


Figure 40: Effect of SABP2 on the binding affinity of SIP470 to TNS. Recombinant SABP2 and SIP470 were incubated together and used for TNS assay. Presence of SABP2 leads to a reduction in TNS fluorescence in the presence of SIP470

CHAPTER 4

DISCUSSION

Salicylic acid (SA) plays an important role in mediating plant defense responses to both biotic and abiotic stress. Plants respond to pathogen attack by producing high amounts of SA which is converted to methyl salicylate; an important lipid mobile signal in plant resistance (Park et al. 2007). Methyl salicylate is converted back to SA by the methylesterase activity of salicylic acid-binding protein 2 (SABP2) to trigger the systemic acquired response (SAR) in plants by increasing the endogenous level of SA in distal plant tissues (Forouhar et al. 2005). This whole process leads to the activation of signaling network through the non-expression of pathogenesis related 1 (NPR1) proteins (Spoel et al. 2003). SA is required for both basal and induced resistance (SAR) response in plants. RNAi-mediated silencing of tobacco SABP2 renders plants defective in inducing SAR (Kumar and Klessig 2003). To learn more about this network involving SABP2, a yeast two-hybrid screening using SABP2 as bait resulted in the identification of several putative SABP2-interacting proteins (SIPs). SIP470 (SABP2 Interacting Protein 470) is one of such protein. Bioinformatics analysis of the nucleotide sequence of SIP470 obtained from the yeast-two hybrid screening predicted it to belong to a non-specific Lipid Transfer Protein 1 subfamily (nsLTP1) (Chapagai 2014). Plant lipid transfer proteins (PLTPs) are a group of small, soluble, basic and highly conserved proteins primarily responsible for the shuttling of phospholipids and other fatty acids between cell membranes. A number of studies reveal that PLTPs play a very important role in the defense against phytopathogens (Maldonado et al. 2002; Jung et al. 2009), cell signaling, antimicrobial defense, cutin synthesis, and cell wall

loosening but these functions have not been well correlated with their in-vitro activity or structure (Yeats and Rose 2008).

In an attempt to determine if SIP470 also plays a significant role in mediating stress responses in plants, *Arabidopsis* T-DNA insertion knockout plants lacking SIP-470 homolog (LTP12 and LTP2) was identified and used for analysis. *Arabidopsis* LTP2 and LTP12 are the most similar proteins to SIP470. Preliminary studies involving these two mutants indicated that they are both defective in growth phenotype as well as susceptible to pathogens (Chapagai 2014). These preliminary findings ignited further interest towards understanding the following questions: 1) what role does SIP470 play in the SA-mediated defense pathway? 2) What role does SIP470 play in biotic and abiotic response in plants? 3) Biochemical characterization of lipid binding activity of SIP470?. To answer these question, we hypothesized that SIP470 is a lipid transfer protein that facilitates the transfer of lipid-like signals during biotic and abiotic stress and thus has a role to play in the biotic and abiotic stress of plants. An attempt to characterize SIP470 based on its lipid transfer activity was not successful in previous studies (Chapagai 2014). The main aim of this study was to determine the possible role of SIP470 and its homolog in *Arabidopsis* during the abiotic and biotic stress as well as its role in the SA-mediated response of plants and also characterize SIP470 in its ability to bind to lipids.

In order to characterize SIP470 as a lipid transfer protein as predicted by the bioinformatics analysis of its coding sequence, a lipid binding assay with minor modifications using a lipophilic probe 2-p-toluidinonaphthalene-6-sulfonate (TNS) was performed (Buhot et al 2004). Generally, proteins are annotated as a lipid transfer protein by the presence of 8 conserved cysteine amino acids which is also present in SIP470 but this does not predict its lipid binding activity because the conserved 8 cysteine motif is not unique to only LTPs and can be found in

several other plant proteins like the proteases and amylase inhibitor as well as in other proteins whose functions are not known (Jose'-Estanyol et al. 2004; Debono et al 2009). To perform the lipid binding assay, soluble recombinant SIP470 without the signal peptide was overexpressed in *E. coli* at 37°C. The recombinant SIP470 protein was then purified using NI-NTA affinity chromatography which removed a number of unwanted proteins. Purified SIP470 was then incubated with TNS (see materials and methods). Under experimental conditions, SIP470 binds to TNS as indicated by the increasing fluorescence as shown in Figure 39. To validate the lipid binding assay, various concentration of SIP470 (250-1250 nM) were used in an assay with a fixed concentration of TNS. Increased fluorescence was observed (data not shown) suggesting that it is the soluble SIP470 that is responsible for the observed fluorescence. This finding confirms that SIP470 is an LTP. This finding is in line with previous findings which suggests that family 1 LTPs have a conserved Tyr79 amino acid that is responsible for intrinsic fluorescence that is observed experimentally and it is normally enhanced upon lipid binding (Lullien-Pellerin et al. 1999). This finding provides evidence which suggests that SIP470 is a lipid binding protein.

To examine the relationship between SABP2 and SIP470, we performed a lipid binding assay in the presence of SABP2. The result from the experiment showed a decrease in the fluorescence of SIP470 in the presence of SABP2 (Figure 40). SABP2 alone showed no fluorescence in the presence of TNS. This finding suggests a possible interaction between SABP2 and SIP470 which affects the ability of SIP470 to bind to TNS. This could provide another evidence that SIP470 is an SABP2 interacting protein.

To test the possible role of SIP470 in biotic stress, T-DNA insertional knockout mutants for LTP12 and LTP2 (SIP470 closest homologs in *Arabidopsis*) were obtained from the

Biological Resource Center, OH, USA, and screened (Chapagai 2014). Pathogen growth assay was performed using these mutants (see methods section). The *ltp12* mutants were more susceptible to virulent pathogens (DC3000) compared to the wild-type Col 0 (figure 30A and Figure 30B) suggesting that they are defective in basal resistance to pathogens. There wasn't a significant difference in the susceptibility of *ltp2* mutants to the virulent pathogen (DC3000) when compared to Col 0 (figure 30C). Collectively, this result suggests that *Arabidopsis ltp12* mutant is a positive regulator of basal resistance or plays a role in protecting the plants from pathogens. To investigate at the molecular level what gene/s might be affected in the knockout *ltp12* mutant plant, we conducted a gene expression studies of plant defense gene (*PR1*) in the presence of both virulent pathogens (see methods section). Results showed that the expression of *PR1* genes was delayed in the *ltp12* mutant plants but not in the wild-type plants on exposure to the virulent pathogen (figure 35). This delay in the induction of *PR1* gene in *ltp12* mutant plants could be the reason they are more susceptible to the virulent pathogen as compared to the wild-type plants. To test if SIP470 has a role to play in the systemic acquired resistance (SAR) of plants, we conducted a study using both *ltp12* and *ltp2* mutants. Results showed that *ltp12* and *ltp2* mutants were not affected in induced resistance (figure 29). Plant defense system is very complex and does not follow a linear pattern (Katagiri 2004), it involves many players such as plants hormones such as SA and JA as well as transcriptional factors (Chen 2002). Studies on *Arabidopsis* revealed that *ltp12* gene is induced upon pathogen attack (Kilian et al. 2007). Similarly, studies on the gene expression studies of SIP470 shows that it is also expressed upon viral and bacterial pathogens (Simo and Kumar unpublished). These various data collectively suggests that SIP470 and its *ltp12* homolog are involved in biotic stress signaling involving innate immunity in plants. This finding has raised the question on what the exact function of

ltp12 in plants? Evidence that a lipid-based signal is necessary for plants to adequately fight against pathogens was revealed through the characterization of several defective lipase-like proteins (EDS1 and PAD4). Eds1 mutants are compromised in basal resistance which is mediated by resistance (R) genes. Interestingly, the weakened local resistance of pad4 is also mediated by same R genes. These genes encode the TIR-NB-LRR-type proteins (Durant 2004). SABP2 was identified as a lipase whose lipase activity is modulated by salicylic acid (Kumar and Klessig 2003). Maybe SIP470 and its *Arabidopsis* homolog ltp12 function in a similar manner to transfer some lipid signals generated by the lipase activity of SABP2 since they are interacting proteins.

In order to fully delineate the full functional potential of SIP470, we subjected its *Arabidopsis* homolog mutant, ltp12 to abiotic stress. Abiotic stresses in general leads to excessive production of reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) which caused extensive tissue damage and also affects photosynthesis (Ciarmiello et al. 2011). Plants normally respond to these stress by antioxidant mechanisms but the response to these stress can be impaired by stresses themselves (Allan and Fluhr 2007). Plants use a complex defense system which involves quite a number of antioxidant stress-related genes that causes a biochemical change in response to such stress (Ciarmiello et al. 2011). Studies also suggest that ROS requires additional molecules along this pathway to amplify and transduce defense signals (Ciarmiello et al. 2011). The ability of plants to respond to abiotic stresses does not follow a linear pathway but involves a very complex integrated circuits involving many other pathways to coordinate a specific response to a given stimulus (Dombrowski 2009). Studies also suggest that there is a similar mechanism by which plants of response to various abiotic stressors (Ciarmiello et al. 2011) because many drought-inducible genes are also induced by exposure to salt stress and cold

stress. Exposure of plants to high salinity (NaCl) creates an ionic stress in addition of disturbing the water balance. Accumulation of salt can affect plasma membrane lipids in the plant cell (Fujii and Zhu 2009). Since various abiotic stressors lead to the induction of a common signaling pathway for abiotic stress, we used different abiotic stressors to study the possible role of *Arabidopsis* LTP12 gene in abiotic stress response using *ltp12* mutants. Germinated seedlings (on MS media) were transferred to a half strength MS media with various concentration of NaCl, ABA, and H₂O₂. At 50mM NaCl, both wild type and *ltp12* mutant did not show much effect (figure 31B). At 150mM NaCl, both *ltp12* mutants were affected, but clearly, *ltp12* mutant showing a more extensive bleaching of its leaves (figure 31C). At 300mM, both *ltp12* and *col 0* showed similar bleaching effect (figure 31D).

In the presence of 3mM H₂O₂, several *ltp12* mutant plants showed bleaching of their leaves but no bleaching effect was noticed in *Col 0* plants (figure 32).

Similarly, 20μM ABA had almost the same effect observed in 3mM H₂O₂ (figure 33). There is, however, a need to repeat this experiment with varying concentration of ABA and H₂O₂ in order to possibly observe a clear phenotypic effect. We also tested the effect of drought on the survival of *ltp12* mutants. Results revealed that *ltp12* mutants failed to recover after prolong drought but *col 0* plants were able to survive (figure 34). Together, this result correlates with several findings that suggest that LTPs are involved in plants response to drought, salt stress and low temperature (Hughes tor-Toro 1992; Molina and Garcia-Olmedo 1993; Trevino and O'Connell 1998). *Arabidopsis* *ltp3* has been reported to be induced during abiotic stress (Seo et al. 2011). The ability of LTPs to be involved in abiotic stress could be linked to their involvement in epicuticular wax layer and cuticle biosynthesis (Clark and Bohnert 1999).

The other aspect of this research was focused on generating transgenic tobacco lines that are silenced in SIP470 expression and overexpressor lines that overexpress SIP470. The generated transgenic lines will be used for complementation studies. Complementation studies using Arabidopsis mutant with functional tobacco SIP470 is necessary to determine if the defective phenotype and the susceptibility to pathogens and abiotic stress could be complemented. This complementation assay would be another way to characterize SIP470. RNAi method was employed in generating our transgenic line that is silenced in the expression of SIP470. Analysis of various independent transgenic lines shows a successful silencing of SIP470 (Figure 27).

Transgenic lines that are overexpressing SIP470 were generated using an inducible system. Generating overexpressor lines was accomplished by cloning the full-length SIP470 into the pER8 vector. Successful clones in pER8 were transiently expressed in *Nicotiana benthamiana* plant (figure 26). Plants normally tend to suppress the expression of foreign lethal proteins, HcPro is a silencing suppressor that helps for successful expression of foreign proteins in plants. From our results (figure 26) it is clear that expression of SIP470 wasn't suppressed in the absence of HcPro. However, transgenic lines for overexpression needs to be screened for an initial trial to screen plants wasn't successful (figure 28)

Future Directions

A significant effort has been put to understand the functional role of SIP470 in plants by using its closest homologs (ltp12 and ltp2) in *Arabidopsis*. So far, we have established that SIP470 homolog, ltp12 mutants are defective in growth phenotype (Chapagai 2014), defective in basal resistance and affected by abiotic stress. We have also established that SIP470 is a lipid transfer protein by its ability to bind to TNS *in vitro*. We also successfully generated transgenic tobacco plants that are silenced in SIP470 and homologs of SIP470 as well as an inducible overexpression line.

To further study the function of SIP470 in plants defense signaling, the generated transgenic plants (silenced and overexpression) should be used for complementation studies to determine if the defect in growth phenotype, as well as susceptibility to pathogens, can be complimented. Similarly, the nature of SABP2 and SIP470 interaction should be pursued to determine they work antagonistically or synergistically in terms of their gene regulation during biotic or abiotic stress. Results from the aforementioned experiments will help enhance our understanding of the functional role of SIP470 in plants.

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APPENDICES

APPENDIX A- Abbreviations

ABA- Absciscic Acid

APS – Ammonium Persulfate

COI1- Coronatine Insensitive 1

Col-0 – Columbia 0

EIN3 - Ethylene Insensitive 3

ET – Ethylene

EtBr – Ethidium Bromide

ETI – Effector Triggered Susceptibility

ETS – Effector Triggered Susceptibility

ICS – Isochorismate Synthase

IPTG - Isopropyl β -D-1-Thiogalactopyranoside

JA- Jasmonic Acid

JAR1 – Jasmonate Resistance 1

KDa – Kilo Dalton

LMW – Low Molecular Weight

LTP12 – Lipid Transfer Protein 12

LTP2 – Lipid Transfer Protein 2

MeJA- Methyl Jasmonate

MeSA – Methyl Salicylate

NB-LRR – Nucleotide Binding Leucine Rich Repeat

Ni-NTA – Nickel-Nitrilotriacetic Acid

nsLTP1 – Non-Specific Lipid Transfer Protein 1

PAL – Phenylalanine Ammonia-Lyase

PAMPS – Pathogen Associated Molecular Pattern

PCR – Polymerase Chain Reaction

PLTP – Plant Lipid Transfer Protein

PR- Pathogenesis-Related

PR1- Pathogenesis-Related Protein 1

PTI – PAMP-Trigged Immunity

RecSIP470 – Recombinant SABP2 Interacting Protein 470

RNAi – RNA Interference

SA – Salicylic Acid

SABP2 - Salicylic Acid Binding Protein 2

SAG – Salicylic Acid beta-Glucoside

SAMT –Salicylic Acid Methyl Transferase

SAR – Systemic Acquired Resistance

SDS – Sodium Dodecyl Sulfate

SDS-PAGE - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SFD – Suppressor of Fatty Acid Desaturase LTPs

SIP – SABP2 Interacting Protein

SIP470 - SABP2 Interacting Protein-470

SOS – Salt Overly Sensitive gene

β -ME - β mercaptoethanol

T-DNA - Transfer DNA

TNS - 2-p-toluidino naphthalene-6-sulfonate

μ g – Microgram

μ l – Microlitre

ng/ μ l – Nanogram/Microliter

APPENDIX B – Buffers and Reagents

1.5 % Agarose Gel

0.75 g of Agarose

50 ml of 1x TAE Buffer

Add the agarose in TAE buffer and heat in the microwave for 60 seconds to dissolve.

Cool the mixture by placing it in a 50°C water bath

Add 2.5 µl (10 mg/mL) ethidium bromide before pouring in gel casting tray.

0.1% DEPC (Diethyl Pyrocarbonate) Treated Water (0.1 L)

0.1 ml of Diethyl pyrocarbonate was added to 100ml of milli-Q water

Mixture was incubated at 37°C for ~12 hours

Then autoclave at 121°C and 15 psi atmospheric pressure for 20 minutes

20% APS (Ammonium Persulfate) (0.5ml)

0.1 g of Ammonium persulfate

0.5 ml of water

Ni-NTA Binding Buffer (1 L)

6.89g of sodium phosphate monobasic (M.W: 137.99 g/mol), final concentration 50 mM

17.53g of Sodium chloride (M.W: 58.44 g/mol), final concentration 300 mM

0.6808g of Imidazole (M.W: 68.08), final concentration 10 mM

Ni-NTA Elution Buffer (1 L)

6.896g sodium phosphate monobasic (M.W: 137.99 g/mol), final concentration 50mM

17.53g sodium chloride (M.W: 58.44 g/mol), final concentration 300 mM

17.02g imidazole (M.W: 68.08), final concentration 250 mM

Western Blotting Blocking Buffer (100 ml)

100 ml 1X PBS buffer

1g Fat-free Milk Powder

3g BSA

10x Western Blotting Transfer Buffer 1L

30.3g tris base (M.W: 121.1 g/mol), final concentration 125 mM

72.06g glycine (M.W: 75.07 g/mol), final concentration 960 mM

Dissolve in 500 ml distilled water and adjust to 1000 ml.

1x Western Blotting Transfer Buffer 1L

100 ml 10x western blotting transfer buffer

100 ml 100% methanol

800 ml cold distilled water

TE Buffer 1L

1.576g tris-HCl (M.W: 157.60 g/mol), final concentration 10 mM/L

Adjust pH to 8

Adjust volume to 1 liter with water

2x SDS-PAGE Loading Dye (100 mL)

10 mL of 1M Tris-Cl, pH 6.8, final concentration 100mM

0.4g SDS, final concentration 0.4% (v/v)

20 ml of glycerol, final concentration 20% (v/v)

0.2 g bromophenol blue, final concentration 0.2% (v/v)

Add 5 mL of β ME before use

10 mM Magnesium Chloride (1 L)

Magnesium Chloride = 0.952 g

Adjust the volume to 1 liter with distilled water

Ni-NTA Binding Buffer (1 L)

6.89 g sodium phosphate monobasic (M.W: 137.99 g/mol), final concentration 50mM/L

17.53 g sodium chloride (M.W: 58.44 g/mol), final concentration 300 mM/L

Imidazole (M.W: 68.08) = 0.6808, final concentration 10 mM/L

Ni-NTA Elution Buffer (1 L)

6.896g sodium phosphate monobasic (M.W: 137.99 g/mol), final concentration 50 mM/L

17.53g sodium chloride (M.W: 58.44 g/mol), final concentration 300 mM/L

17.02 g imidazole (M.W: 68.08), final concentration 250 mM/L

LB Medium (1 L)

25g LB Broth

Make up to 1000 ml with distilled water

Sterilize by autoclaving for 15 minutes on liquid cycle

Store at 4°C temperature

Lipid Binding Assay Buffer 100ml

175 mM mannitol

0.5 mM K₂SO₄

0.5 mM CaCl₂,

5 mM MES,

Adjust pH to 7.0

SOC MEDIA 100ml

80ml LB media

Add 20ml of 1M glucose

Autoclave and store at 4°C

STE Buffer 50ml

100mM NaCl

10mM Tris-HCl,

Adjust to pH 8

1mM EDTA

STET solution 50ml

10mM Tris-HCl

1mM EDTA

100mM NaCl

5% Triton X-100

Half Strength MS media

2.155g/L of MS salt

Add 1% sucrose

Adjust pH to 5.9 using 1M KOH

Add 0.9% Agar.

Autoclave

1X PBS (1 L)

100 ml 10X PBS

900 ml distilled water

1X PBS Plus 3% Tween Twenty (1 L)

100 ml 10X PBS

870 ml distilled water

30 ml tween 20

Shoot Inducing Media (SIM) 1L

4.31g of MS salts

Add 3% Sucrose

Adjust pH to 5.9 with 1M KOH

Add 0.9% Agar

Add Vitamins

+0.1mg/litre alpha NAA

+1mg/litre BAP

+1ml 1000X vitamin solution

SIM with antibiotics

To SIM media, add:

Carbenicillin 250mg/litre

Kanamycin 300mg/litre

Cefatoxim 100mg/ml

Root Inducing Media (RIM)

4.31g of MS salts

Add 1% Sucrose

Adjust pH to 5.9 with 1M KOH

Add 0.9% Agar

Add Vitamins

+0.1mg/litre alpha NAA

+1mg/litre BAP

+1ml 1000X vitamin solution

APPENDIX C – Sequence Analysis for Silencing and Homolog Silencing

Sequence Analysis for Silencing SIP470





BLAST

Input parameters

Help

Categories

N.tabacum Genomes (Current version)

Database

N.tabacum K326 CDS

db details

Program

blastn (nucleotide to nucleotide db)

Query

autodetect

Show example

```

ATGGAAATGGTTGGCAAGATTGCGTGCTTTGTGTTACTTTGCATGGTGGTGGTTGCACCCCATGCAGAGGCACTGAGCTGCGGCCAGGTTTCAGT
CTGGCCTGGCTCTTTGCCTCCCTTATCTGCAGAGTCGCGGCCCTCTAGGGAGGTGTTGTGGCGGCCTTAAAGGTGTGTTGGGTGCTGCCCCG
ACCCAGCTGACCGCAAGACTGCATGCACTTGCCTGAAATCAGCTGCTTATGCTATTAAGGGTATTAATATGGGCAAAGCCGCTGGACTTCCTAGT
GCTTGTGGCGTTAACATTCCTACAAGATCAGTCCCTCTACTGACTGCTCCAGGGTCCAGTGA
          
```

BLAST

Advanced Options

Untitled_sequence vs N.tabacum K326 CDS

SubjectId	id%	Aln	evalue	Score	Description
mRNA_37947_cds	100.00	345/345	0.0	684	mRNA_37947 gene_21929 id=AT2G38530.1:evalue=3e-28;annot='lipid transfer protein 2';id=Solyc10g075070.1.1:evalue=3e-63;annot='Non- specific lipid-transfer protein' Length = 489
mRNA_102807_cds	89.28	308/345	e-108	391	mRNA_102807 gene_60821 id=AT2G38530.1:evalue=1e-28;annot='lipid transfer protein 2';id=Solyc10g075070.1.1:evalue=9e-68;annot='Non- specific lipid-transfer protein' Length = 345
mRNA_87105_cds	88.05	302/343	3e-97	355	mRNA_87105 gene_51017 id=AT2G38540.1:evalue=3e-28;annot='lipid transfer protein 1';id=Solyc10g075070.1.1:evalue=9e-66;annot='Non- specific lipid-transfer protein' Length = 345
mRNA_30771_cds	88.05	302/343	3e-97	355	mRNA_30771 gene_17748 id=AT2G38540.1:evalue=2e-26;annot='lipid transfer protein 1';id=Solyc10g075070.1.1:evalue=2e-65;annot='Non- specific lipid-transfer protein' Length = 345
mRNA_37999_cds	87.83	303/345	5e-96	351	mRNA_37999 gene_21951 id=AT2G38540.1:evalue=3e-26;annot='lipid transfer protein 1';id=Solyc10g075110.1.1:evalue=6e-62;annot='Non- specific lipid-transfer protein' Length = 489

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>mRNA_30771

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>mRNA_37999

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AAATGGTAAGCAAGATTGCATGTTTCGTGGTTTTGTGCATGGTGGTGGTTGCACCCCATGCAGAGGCACTGACC
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TGGCGGCGTTAAACGTCTGTTGGGTGCTGCTCGCACCCAGCGGACCGAAAGACTGCATGCAATTGCCTGAAAT
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CCCTACAAGATCAGTCCCTCCACTGACTGCTCCAAGGTCCAGTGA

>SIP470

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AGGGAGGTGTTGTGGCGGCGTTAAAGGTGTGTTGGGTGCTGCCCCGACCCCACTGACCGCAAGACTGCA
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CLUSTAL O(1.2.2) multiple sequence alignment

```

mRNA_30771_cds      CTTTTAAGAATGGAAATGGTAAGCAAGATTGCATGTTTCGTGGTTTTGTGCATGGTGGTG
SIP470              -----ATGGAAATGGTTGGCAAGATTGCGTGCTTTGTGTTACTTTGCATGGTGGTG
mRNA_37999_cds      CTTTTAAGAATGGAAATGGTAAGCAAGATTGCATGTTTCGTGGTTTTGTGCATGGTGGTG
mRNA_102807_cds     -----ATGGAAATGGTAGGCAAGATTGCATGTTTCGTGGTTTTGTGCATGGTGGTG
mRNA_87105_cds      -----ATGGAAATGGTAGGTAAGATTGCATGCTTTGTGGTTTTGTGCATGGTGGTG
                      *****:.* *****.* * * * * *: * *****

mRNA_30771_cds      GTTGCACCCCATGCAGAGGCACTGACCTGCGGCCAGGTTCACTCTAGCCTGGCTCCTTGC
SIP470              GTTGCACCCCATGCAGAGGCACTGAGCTGCGGCCAGGTTCACTCTGGCTGGCTCCTTGC
mRNA_37999_cds      GTTGCACCCCATGCAGAGGCACTGACCTGCGGCCAGGTTCACTCTAGCCTGGCTCCTTGC
mRNA_102807_cds     GTTGCACTCATGCAGAGGCACTAAGCTGCGGCCAGGTTCACTCTGGCTGGCTCCTTGC
mRNA_87105_cds      GTTGCACCCCATGCAGAGGCACTGAGCTGCGGCCAAGTTCACTCCGCTGGCTCCTTGT
                      ***** *****.* *****.* *****.* *****

mRNA_30771_cds      GTCCCTTATTTGCTGGGCCGCGGCCCTTTGGGGGGTTGTTGTGGCGCGTTAAACGCTCTG
SIP470              CTCCCTTATCTGCAGAGTCGCGGCCCTTAGGGAGGTGTTGTGGCGCGTTAAAGGTGTG
mRNA_37999_cds      GTCCCTTATTTGCTGGGCCGCGGCCCTTTGGGGGGTTGTTGTGGCGCGTTAAACGCTCTG
mRNA_102807_cds     CTCCCTTATCTACAGGGCGTGCCCTCTCGGAGGCTGTTGTGGTGGTGTAAAGGTCTG
mRNA_87105_cds      CTCCCTTATTTGCAGGGCCGCGGCCCTTTGGGGAGCTGTTGTGGTGGTGTAAAGGTCTG
                      ***** *.*:.* * * ***** * *.*: ***** * * * *

mRNA_30771_cds      TTGGGTGCTGCTCGCACCCAGCGGACCGAAAGACTGCATGCAATTGCCTGAAATCAGCC
SIP470              TTGGGTGCTGCCCCGACCCAGCTGACCGCAAGACTGCATGCACTTGCTGAAATCAGCT
mRNA_37999_cds      TTGGGTGCTGCTCGCACCCAGCGGACCGAAAGACTGCATGCAATTGCCTGAAATCAGCC
mRNA_102807_cds     TTAGGTGCAGCCAAGTCCCCAGCTGACCGCAAGACTGCATGCACTTGCTGAAATCGGCC
mRNA_87105_cds      TTGGGTGCAGCCAAGTCCCCATCTGACCGGAAGACTGCATGCACTTGCTGAAATCGGCT
                      *.*****:.* .. :***** * ***** *****.******.*

mRNA_30771_cds      GCTAATACTTTTAAGGGCATTGATATGGGCAACGCCGCTCGTCTCCCTGGTACTTGTGGC
SIP470              GCTTATGCTATTAAGGGTATTAATATGGGCAAAGCCGCTGGACTTCCTAGTGCTTGTGGC
mRNA_37999_cds      GCTAATACTTTTAAGGGCATTGATATGGGCAACGCCGCTCGTCTCCCTGGTACTTGTGGC
mRNA_102807_cds     GCTAATGCTATTAAGGGTATTGACATGGGCAAAGCCGCTGGACTCCCTAGTGCTTGTGGC
mRNA_87105_cds      GCTAATGCTATTAAGGGTATTGATATGGGCAAAGCCGCTGGTCTCCCTGGTGCTTGTGGT
                      ***:*.**.*:***** *.* *****.****** *:** ***.**.******

mRNA_30771_cds      GTTAACATTCCCTACAAGATCAGTCCCTCCACTGACTGCTCCAAGGTCCAGTGA
SIP470              GTTAACATTCCCTACAAGATCAGTCCCTCTACTGACTGCTCCAGGTCCAGTGA
mRNA_37999_cds      GTTAACATTCCCTACAAGATCAGTCCCTCCACTGACTGCTCCAAGGTCCAGTGA
mRNA_102807_cds     GTTAACATTCCATAACAAGATCAGTCCCTCCACTGATTGCTCCAAGGTCCAGTGA
mRNA_87105_cds      GTCAACATTCCCTACAAGATCAGCCCCCTCCACAGACTGCTCTAAGGTCCAGTAA
                      * * ***** ***** ***** **:* * * * * *.*****.*

```

The sequence in red is the unique 60bp sequence used for gene silencing.

mRNA_30771_cds SIP470	CTTTTAAGAATGGAAATGGTAAGCAAGATTGCATGTTTCGTGGTTTTGTGCATGGTGGTG -----ATGGAAATGGTTGGCAAGATTGCGTGCTTGTGTTACTTTGCATGGTGGTG
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mRNA_102807_cds	-----ATGGAAATGGTAGGCAAGATTGCATGTTTCGTGGTTTTGTGCATGGTGGTG
mRNA_87105_cds	-----ATGGAAATGGTAGGTAAGATTGCATGCTTGTGGTTTTGTGCATGGTGGTG *****:.* *****.* ** * ** *: * *****
mRNA_30771_cds SIP470	GTTGCACCCCATGCAGAGGCACTGACCTGCGGCCAGGTTTCAGTCTAGCCTGGCTCCTTGC GTTGCACCCCATGCAGAGGCACTGAGCTGCGGCCAGGTTTCAGTCTGGCTGGCTCCTTGC
mRNA_37999_cds	GTTGCACCCCATGCAGAGGCACTGACCTGCGGCCAGGTTTCAGTCTAGCCTGGCTCCTTGC
mRNA_102807_cds	GTTGCACCTCATGCAGAGGCACTAAGCTGCGGCCAGGTTTCAGTCTGGCTGGCTCCTTGC
mRNA_87105_cds	GTTGCACCCCATGCAGAGGCACTGAGCTGCGGCCAAGTTTCAGTCCGGTTGGCTCCTTGT ***** *****.* *****.* ***** .** ***** **
mRNA_30771_cds SIP470	GTCCCTTATTTGCTGGGCCGCGGCCCTTTGGGGGGTTGTTGTGGCGCGTTAAACGCTCTG CTCCCTTATCTGCAGAGTCGCGGCCCTCTAGGGAGGTGTTGTGGCGCGTTAAAGGTGTG
mRNA_37999_cds	GTCCCTTATTTGCTGGGCCGCGGCCCTTTGGGGGGTTGTTGTGGCGCGTTAAACGCTCTG
mRNA_102807_cds	CTCCCTTATCTACAGGGCGTGCCCTCTCGGAGGCTGTTGTGGTGGTTAAAGGTCTG
mRNA_87105_cds	CTCCCTTATTTGCAGGGCCGCGGCCCTTTGGGGAGCTGTTGTGGTGGTTAAAGGTCTG ***** *.*:.* ** ***** * **.* ***** ** ***** ** **
mRNA_30771_cds SIP470	TTGGGTGCTGCTCGCACCCAGCGGACCGAAAGACTGCATGCAATTGCCTGAAATCAGCC TTGGGTGCTGCCCCGACCCAGCTGACCGCAAG ACTGCATGCACTTGCCTGAAATCAGCT
mRNA_37999_cds	TTGGGTGCTGCTCGCACCCAGCGGACCGAAAGACTGCATGCAATTGCCTGAAATCAGCC
mRNA_102807_cds	TTAGGTGCAGCCAAGTCCCCAGCTGACCGCAAGACTGCATGCACTTGCCTGAAATCGGCC
mRNA_87105_cds	TTGGGTGCAGCCAAGTCCCCATCTGACCGGAAGACTGCATGCACTTGCCTGAAATCGGCT **.****:.* .. :***** * ***** **********.*****.*
mRNA_30771_cds SIP470	GCTAATACTTTTAAGGGCATTGATATGGGCAACGCCGCTCGTCTCCCTGGTACTTGTGGC GCTTATGCTATTAAGGGTATTAATATGGGCAAAGCCGCTGGACTTCCTAGTGCTTGTGGC
mRNA_37999_cds	GCTAATACTTTTAAGGGCATTGATATGGGCAACGCCGCTCGTCTCCCTGGTACTTGTGGC
mRNA_102807_cds	GCTAATGCTATTAAGGGTATTGACATGGGCAAAGCCGCTGGACTCCCTAGTGCTTGTGGC
mRNA_87105_cds	GCTAATGCTATTAAGGGTATTGATATGGGCAAAGCCGCTGGTCTCCCTGGTACTTGTGGT ***:.*.*:***** **.* *****.* ***** *:.* **.*.*.*****
mRNA_30771_cds SIP470	GTTAACATTCCCTACAAGATCAGTCCCTCCACTGACTGCTCCAAGGTCCAGTGA GTTAACATTCCCTACAAGATCAGTCCCTCTACTGACTGCTCCAGGGTCCAGTGA
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mRNA_102807_cds	GTTAACATTCCATACAAGATCAGTCCCTCCACTGATTGCTCCAAGGTCCAGTGA
mRNA_87105_cds	GTCAACATTCCCTACAAGATCAGCCCCCTCCACAGACTGCTCTAAGGTCCAGTAA ** ***** ***** ***** **.* **.* *****.* *****.*

The sequence in red is the unique 141bp sequence used for Homolog silencing.

VITA

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- Audam T, and Kumar D. (2015) *Characterization of SIP470, a
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